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(54) Title: MONOCLONAL ANTIBODIES TO LEUKOCYTE ADHESION MOLECULE-1

(57) Abstract

A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-1, -2, -4, -5, -6, -7, -8, -9, -10, -11, -14 or -15; the hybridoma cell which produces such monoclonal antibody; and methods of using such monoclonal antibody.

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MONOCLONAL ANTIBODIES TO LEUKOCYTE ADHESION MOLECULE-1

This application is a continuation-in-part of Tedder et al., U.S.S.N. 07/720,602, filed June 25, 1991, and Tedder, U.S.S.N. 07/700,773, filed May 15, 1991, both of which are continuations-in-part of Tedder, U.S.S.N. 07/313,109, filed February 21, 1989, each of which is herein incorporated by reference.

Part of the work leading to this invention was made with funds provided under grants AI 26872 and CA 34183 by the United States Government, which has certain rights in this invention.

This invention relates to human leukocyte-associated cell surface proteins, and monoclonal antibodies specific for such proteins.

Background of the Invention

Genes exclusively expressed by one cell lineage, but not by others, often define the function of that cell population. The generation of genes by the assembly of functionally independent domains has occurred frequently as new genes have evolved to encode proteins with new functions. An inducible endothelial-leukocyte adhesion molecule (ELAM-1), having several functionally independent domains, is expressed on the surface of cytokine-treated endothelial cells. This molecule is thought to be responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of cells to the vascular lining (Bevilacqua et al., Proc. Natl. Acad. Sci. USA 84:9238 (1987)). A granule membrane protein found

in platelets and endothelial cells, termed GMP-140, has been cloned and is homologous with ELAM-1 (Johnston et al., Blood Suppl. 1 72:327A (1988)).

Summary of the Invention

The invention generally features a leukocyte-associated cell surface protein LAM-1 (leukocyte adhesion molecule-1), which contains domains homologous with binding domains of animal lectins, growth factors, and C3/C4 binding proteins; the specific domains of the LAM-1 protein; and the genomic DNA sequences encoding the LAM-1 protein and the specific domains of LAM-1.

Preferred embodiments of the invention include essentially purified proteins comprising sequences of amino acids having 90% or greater homology with, or identity with, the amino acid residues of specific domains of human leukocyte-associated cell surface protein LAM-1 represented in Fig. 2, i.e., the lectin domain represented by residues 42-170, the EGF-like domain represented by residues 171-206, the short consensus repeat unit I domain represented by residues 207-269, the short consensus repeat unit II domain represented by residues 270-331, the leader domain represented by residues 15-41, the transmembrane domain represented by residues 332-373, and the phosphorylation domain represented by residues 374-380.

Also within the invention is a monoclonal antibody which recognizes (i.e., binds to, with immunologic specificity) the LAM-1 epitope recognized by any one of the monoclonal antibodies designated anti-LAM1-1, -2, 4, -5, -6, -7, -8, -9, -10, -11, -14 OR -15. Each such epitope is defined by its binding to a particular anti-LAM1 monoclonal antibody. Thus, whether or not a given antibody is within th invention may be determin d by comparison of its epitop to that of the stated anti-LAM1 mAb. This comparis n may be

done by any method or combination of methods for such determinations generally known to immunologists, including the following:

1. A recombinant gene encoding a chimeric protein composed of one of the LAM-1 functional domains (e.g., the lectin domain) fused to the other domains (e.g., the EGF-like and SCR domains) from another selectin could be used to assay for binding by the stated anti-LAM1 mAb and by the antibody to be tested. If one binds to the chimeric protein while the other does not, the two mAbs have different epitopes.

2. An antibody binding to its epitope can block the subsequent binding of a different antibody recognizing the same or a physically proximal epitope. Thus, if a first antibody bound to its epitope blocks the binding of a second antibody, the two are likely to recognize the same epitope.

3. LAM-1 functions are carried out by specific regions of the molecule, which are composed of individual epitopes. Therefore, an epitope involved in ligand binding, for example, can be identified by the ability of an antibody bound to that epitope to inhibit a given LAM-1 function, such as the binding of LAM-1 to PPME, fucoidin, HEV, or activated endothelial cells. Also, the binding of an antibody to its epitope may completely or only partially inhibit the given LAM-1 function, depending on the proximity of the epitope to the ligand binding site of the protein, so that the epitopes of two different antibodies may be distinguished on the basis of the difference in degree of inhibition by the two antibodies.

4. Some epitopes of LAM-1 may also be involved in the regulation of function of the molecule, such that antibody binding t those epitopes may augment a given LAM-1 functi n. F r example, antibody binding to the LAM1-1 or

LAM1-5 epitope increases the level of PPME and fucoidin binding by LAM-1.

5. Some LAM-1 epitopes may be conformationally determined, and would therefore be expressed on the LAM-1 protein to different degrees under identical conditions. The level of reactivity of two different antibodies for the same epitope would be expected to vary identically as the conditions of immunological assay are varied, thus providing a means for determining whether one antibody reacts to the same epitope as another.

6. Since epitopes are determined by primary amino acid sequence of the LAM-1 protein, subtle differences in amino acid sequence in specific regions of the protein, such as occurs during evolution of species, will alter structure of the epitope, and may therefore alter antibody binding to that epitope, without affecting binding of a different antibody to a different epitope on the same molecule. Therefore, different patterns of reactivity of the antibodies to the LAM-1 proteins of different animal species can be used to identify distinct epitopes.

The above assays for the ability of a given antibody to bind to the epitope of a second antibody are described in the Detailed Description set forth below.

The invention includes anti-LAM1-1, -2, -4, -5, -6, -7, -8, -9, -10, -11, -14, and -15 and the hybridoma cells which produce any of the mAb of the invention. Each of the mAb has been deposited with the American Type Culture Collection (ATCC).

The mAb of the invention may be used in a method of identifying cells expressing LAM-1 (i.e., bearing LAM-1 molecules on their surface), which method includes the steps of providing a sample of cells (e.g., from an animal or a cell line), at least some f which are suspected of

expressing LAM-1; contacting the cells with a reagent which includes the mAb; and determining which cells form an immune complex with the reagent. This reagent may be the mAb alone (e.g., in the form of a purified preparation or unfractionated ascites fluid), or may be the mAb conjugated with a detectable label (such as a detectable enzyme, fluorophore, or radioactive moiety). Determination of which cells form an immune complex with the reagent may be accomplished by any of a variety of standard immunological techniques, including immunoprecipitation, indirect or direct immunofluorescence with flow cytometry analysis, or immunosorbent assays.

In addition, the mAb of the invention may be used in a method of isolating cells expressing LAM-1, which method includes the steps of providing a sample of cells, at least some of which are suspected of expressing LAM-1; contacting the cells with the mAb or a reagent including the mAb; and separating those cells which have formed an immune complex with the mAb, from those cells which have not. This separation may be performed by any of a variety of standard immunological techniques, including fluorescence-based cell sorting, magnetic bead-based separation protocols, and the use of solid phase bound antibody.

The mAb of the invention is also useful in a diagnostic assay for detecting leukocyte activation in an animal, which activation may be attributable, for example, to inflammation, an autoimmune response, or rejection of an organ or tissue transplant. This method is accomplished by obtaining a fluid sample from an animal (preferably a human), which sample may be, for example, blood, serum, plasma, saliva, tears, cerebral spinal fluid, or urine; contacting the sample with the mAb of the invention; and detecting (by standard immunological techniques) formation

of immune complexes by the mAb, which immune complexes are indicative of the presence of LAM-1 shed from leukocytes, an indicator of leukocyte activation.

The mAb of the invention is particularly useful for blocking leukocyte interactions with endothelium of an animal, which method involves administering an effective amount of the mAb, or a therapeutic which includes the mAb or a LAM-1-blocking portion of the mAb, to the animal. This LAM-1-blocking portion of the mAb may be, for example, a F(ab) fragment, and the mAb may be part of a chimerized antibody having a variable region derived from a mAb of the invention, and constant regions derived from a human antibody. The animal (preferably a human) may be suffering from inflammation, an autoimmune response, rejection of an organ or tissue transplant, or any other condition involving leukocyte interactions with endothelium.

Also within the invention is a method of determining the degree of expression of LAm-1 in a sample of leukocytes (i.e., the amount of LAM-1 expressed by the cells, determined, for example, as a quantitative amount per cell or per sample, as a percentage of a standard amount, as the percentage of cells which have over or under a certain amount, or perhaps just as relatively more or less than a standard sample of cells), which method includes the steps of contacting a sample of leukocytes with the mAb of the invention, and determining the level of immune complex formation in the sample, such level being indicative of the degree of expression of LAM-1 on the leukocytes.

In another aspect the invention features methods of treating a patient suffering from a leukocyte-mobilizing condition (e.g., tissue damage, an autoimmune disease, or cancer), or who is an organ or tissue transplanted

recipient, which method includes administering to the patient a therapeutic agent including a therapeutic amount of the LAM-1 protein or a domain thereof, or of an antagonist to the LAM-1 protein or domain thereof, or of a fusion protein including the LAM-1 protein or a domain thereof covalently bonded to an immunoglobulin heavy chain constant region. The therapeutic agent is administered in a pharmaceutically acceptable carrier substance.

In another aspect, the invention features using the LAM-1 protein or domain thereof to identify a ligand that binds to the protein or to a molecule that is specifically associated with the protein, or fragment thereof, to generate a functional molecule. Ligands so identified can also be used in the methods of the invention described above.

As used herein the term "antagonist to LAM-1" includes any agent which interacts with LAM-1 and interferes with its function, e.g., antibody reactive with LAM-1 or any ligand which binds to LAM-1. The term "identify" is intended to include other activities that require identification of an entity, such as isolation or purification. The term "essentially purified" refers to a protein or nucleic acid sequence that has been separated or isolated from the environment in which it naturally occurs.

Leukocyte-associated cell surface protein LAM-1 plays an important role in leukocyte-endothelial cell interactions, especially selective cell trafficking to sites of inflammation. The LAM-1 protein or domains thereof, or other molecules that interfere with leukocyte adhesion and function, can be used therapeutically to inhibit the inflammatory response and to treat such conditions as tissue damage and m tastasis of cancer cells.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Description of the Preferred Embodiments

In the drawings, Figs. 1A and 1B show the structure of LAM-1 cDNA clone.

Fig. 2 shows the cDNA nucleotide sequence and the predicted amino acid sequence of LAM-1.

Figs. 3A, 3B, and 3C show the homologies of LAM-1 with other proteins.

Fig. 4 depicts the immunoprecipitation of LAM-1 shed from a cell surface with anti-LAM1 antibodies or a control immunoprecipitation with an unreactive isotype-matched antibody with subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

Fig. 5 depicts the percentage and reactivity of malignant cells being LAM-1 positive from patients having various forms of hematopoietic malignancies;

Fig. 6 depicts the immunoprecipitation of LAM-1 from the surface of iodinated CLL cells using anti LAM-1 antibodies or an unreactive isotype-matched control with subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reduced and non-reduced conditions;

Fig. 7 depicts indirect immunofluorescence results obtained with the anti-LAM-3 antibody and PPME-FITC;

Fig. 8 depicts the modulation of cell surface LAM-1 by malignant cells and cDNA transfected cells after PMA exposure.

Fig. 9 depicts expression of LAM-1 epitopes by lymphocytes and neutrophils. Cells were examined by indirect immunofluorescence analysis for the expression of LAM-1 epitopes identified by the anti-LAM1-3, anti-LAM1-6, anti-LAM1-7 or anti-LAM1-10 mAb. The relative fluorescence

intensity of staining with each mAb was rated on a (+) to (++++) scale as shown on a three decade log scale. The background staining obtained using an unreactive isotype-matched mAb was less than 5% as shown by the shaded histograms in the first panels.

Fig. 10 depicts modulation of PPME binding by anti-LAM-1 mAb. Flow cytometry analysis of PPME-FITC binding to human blood mononuclear cells after treatment of the cells with medium or anti-LAM-1 mAb 20 min prior to PPME-FITC staining. PPME staining is completely inhibited by 5 mM EDTA (shaded panel), indicating that all of the PPME binding by PBMC is due to LAM-1. This is confirmed by the observation that inhibition by LAM1-3 also reduces the level of PPME staining to background.

Fig. 11 depicts evolutionary conservation of the LAM1-3 epitope. Blood mononuclear cells from a human, cow, rhesus monkey, dog, cat and rabbit were examined in indirect immunofluorescence assays for expression of the LAM-1 epitope identified by the anti-LAM1-3 mAb (dark line). The fluorescence histogram of cells treated with an unreactive murine IgG_1 mAb are also shown (thin line). Cells were examined by flow cytometry analysis and relative fluorescence intensity of staining is shown on four or three decade scales as indicated. Cell samples were examined at different times with different flow cytometer settings so that individual histograms are not directly comparable.

Fig. 12 depicts conservation of the PPME binding receptor by human, tamarin, dog, and rabbit blood mononuclear cells. Cells were incubated with PPME-FITC (3 μg/ml) in medium, medium containing saturating concentrations of the anti-LAM1-3 or anti-LAM1-4 mAb or medium c ntaining 5 mM EDTA. Relative fluorescence

intensity of the stained cells was analyzed by flow cytometry and the percentage of cells with positive staining from each treatment is shown.

Fig. 13 depicts hypothetical model for the functionally and structurally related regions of the LAM-1 protein. Shaded circles indicate the groups of mAb-defined epitopes with similar functional characteristics. Numbers refer to the anti-LAM-1 mAb designations. Additional unshaded circles indicate regions without known functional activity that were localized by domain mapping and mAb cross-blocking studies to different regions of the protein.

Leukocyte migration is regulated principally at the level of interactions between circulating leukocytes and the endothelium. Several adhesion molecules that mediate leukocyte interactions with specialized endothelial cells have been identified (Stoolman *Cell*, 56:907, 1989; Duijvestijn et al., *Immun. Today* 10:23, 1989; Berg et al., *Immunol. Rev.* 108:5, 1989). Leukocyte Adhesion Molecule-1 (LAM-1, TQ-1, Leu-8, LEC-CAM-1), the human homologue of the mouse MEL-14 antigen, mediates the binding of blood leukocytes to high endothelial venules (HEV) of peripheral lymph nodes. LAM-1 is expressed on the surface of human peripheral lymphocytes, neutrophils, eosinophils, monocytes and hematopoietic progenitor cells (Pilarski et al., J. Immunol. (in press); Kansas et al., J. Immunol. 134:2295, 1985; Kansas et al., J. Immunol. 134:3003, 1985; Kansas et al., J. Immunol. 142:3058, 1989; Griffin et al., J. Immunol. 145:576, 1990; Jutila et al., Blood 76:178, 1990), suggesting that it may also serve a more general role in mediating leukocyte interactions with endothelium. Both lymphocyt s and neutrophils express a singl sp ci s of LAM-1 protein (Kansas et al., Blood 76:2483, 1990), but the Mr of cell-surface LAM-1 on lymphocyt s is 74,000 (Tedder et

al., Eur. J. Immunol. 20:1351, 1990) and that of neutrophil LAM-1 is 90-100,000 (Kansas et al., J. Immunol. 142:3058, 1989). This presumably results from different patterns of post-translational processing as occurs with many molecules expressed by both cell lineages. LAM-1 affinity for ligand is transiently increased following leukocyte activation with lineage-specific agents, which may partially explain the differences in migration between neutrophils and lymphocytes (Spertini et al., Nature 349:691, 1991). Among lymphocytes, LAM-1 is expressed by the majority of circulating T and B cells, is lost following mitogen stimulation, but is found on some antigen-specific memory T cells (Tedder et al., Eur. J. Immunol. 20:1351, 1990; Kanof et al., J. Immunol. 140:3701, 1988). LAM-1 is a member of the selectin family of cellular adhesion/homing receptors, which play important roles in leukocyte-endothelial cell interactions, especially selective cell trafficking to sites of inflammation. Like other members of this family, LAM-1 contains an amino-terminal lectin-like domain, followed by an epidermal growth factor (EGF)-like domain and short consensus repeat units (SCR) similar to those found in C3/C4 binding proteins. The lectin domain appears to interact with specific glycoconjugates expressed on high endothelial venules (HEV) of peripheral lymph nodes. This interaction is calcium dependent and can be inhibited by mannose 6-phosphate or mannose 6-phosphate rich polysaccharides, such as the phosphomannan monoester fragment, PPME. PPME is particularly useful as a soluble ligand since it can be labelled and used to assess LAM-1 binding activity without the influence of other adhesion molecules inv lved in l ukocyte binding (Spertini et al., Nature 349691, 1991; Spertini et al., Leukemia (in press) 1991).

Human and murine lymphocytes bind with a similar specificity to HEV of human or murine peripheral lymph node tissue sections, suggesting that murine and human MEL-14/LAM-1 ligands share some evolutionarily conserved structure or epitope(s). This is consistent with the ability of both human and mouse lymphocytes to bind PPME and the ability of PPME to inhibit lymphocyte adhesion to HEV in both species. Consistent with this functional conservation, human LAM-1 and murine MEL-14 are 77% conserved at the amino acid sequence level (Tedder et al., J. Exp. Med. 170:123, 1989). Within the lectin and EGF-like domains, the level of homology increases to 83% and 80% respectively. This high degree of homology makes it likely that the regions of the molecule most critical for function would be the most conserved. In this study, a large panel of mAb reactive with LAM-1 was developed and used to characterize the function of distinct LAM-1 epitopes. Analysis of numerous animal species with these mAb indicates that many of the functionally significant epitopes were also well conserved through recent mammalian evolution, with certain exceptions including mouse LAM-1.

Cloning and characterization of cDNA encoding LAM-1

B cell-specific cDNAs were isolated from a human tonsil cDNA library (ATCC #37546) using differential hybridization with labeled cDNAs derived from either B cell (RAJI) RNA or T cell (HSB-2) RNA (Tedder et al., Proc. Natl. Acad. Sci. USA 85:208-212 (1988)). Positive plaques were isolated and cloned, and the cDNA inserts were subcloned into the plasmid pSP65 (Promega, Madison, WI). Nucleotide sequences were determin d using the method of Maxam and Gilbert (Meth. Enzymol. 65:499 (1980)). Gap penalti s of -1 were assessed during homology analysis for each nucleotide or amino acid in the sequence where a gap or deletion

occurred. One of the 261 RAJI+ HSB2- cDNA clones isolated, B125, contained a 1.90 kb cDNA insert that hybridized with a 2.4 kb RNA species found in several B cell lines (Tedder et al., supra). However, B125 did not hybridize with any of the other RAJI+ RSB2- clones or with mRNA from several T cell lines. The B125 cDNA clone was characterized by restriction mapping and nucleotide sequence determination. A near-full-length 2.3 kb cDNA that hybridized with B125 was isolated, sequenced, and termed pLAM-1.

As shown in Fig. 1A, a restriction map was constructed by the standard single, double or triple digestions of pLAM-1. The coding region is shown in black. Arrows indicate the direction and extent of nucleotide sequence determination and the open circles indicate 5'-end labeling. In Fig. 1B, a schematic model of the structure of the LAM-1 mRNA is shown. Thin lines indicate 5' and 3' untranslated sequences (UT), while the thick bar indicates the translated region. The boxes represent the lectin-like and epidermal growth factor (EGF)-like domains and the two short consensus repeat (SCR) units. The open box indicates the transmembrane (TM) region.

pLAM-1 contains a 1,181 bp open reading frame that could encode a protein of 372 amino acids, as shown in Fig. 2. The numbers shown above the amino acid sequence designate amino acid residue positions. The numbers to the right indicate nucleotide residue positions. Amino acids are designated by the single-letter code, and * indicates the termination codon. The boxed sequences identify possible N-linked glycosylation sites. Hydrophobic regions that may identify signal and transmembrane peptides are underlined. The amino acid sequence of LAM-1 indicates a structure typical f a membrane glycoprotein. The matur LAM-1 pr tein has an extracellular region of about 294 amino

acids containing 7 potential N-linked carbohydrate attachment sites. LAM-1 has a cytoplasmic tail of 17 amino acids containing 8 basic and 1 acidic residues. The processed LAM-1 protein has a M_r of at least 50,000 and can be isolated by conventional techniques, such as affinity column chromatography with antibody or ligand, from cell lines that normally express this receptor or from transfected cell lines. Alternatively, the protein can be synthesized by in vitro translation of the LAM-1 cDNA.

LAM-1 combines domains homologous to domains found in three distinct families of molecules: animal lectins, growth factors, and C3/C4 binding proteins. The extracellular region of LAM-1 contains a high number of Cys residues (7%) with a general structure as diagrammed in Fig. 1B. As indicated in Fig. 3, segments of homologous proteins are shown with the amino acid residue numbers at each end. Homologous amino acids are shown in boxes. Gaps (-) have been inserted in the sequences to maximize homologies. The first 157 amino acids of the protein (Fig. 3A) are homologous with the low-affinity receptor for IgE (Kikutani et al., Cell 47:657 (1986)), the asialoglycoprotein receptor (Spiess et al., Proc. Natl. Acad. Sci. USA 82:6465 (1985)) and several other carbohydrate-binding proteins (Drickamer et al., J. Biol. Chem. 256:5827 (1981); Ezekowitz et al., J. Exp. Med. 167:1034 (1988); Krusius et al., J. Biol. Chem 262:13120-13125 (1987); and Takahashi et al., J. Biol. Chem. 260:12228 (1985)). The amino acids conserved among all animal-lectin carbohydrate recognition domains are indicated (*). Although the sequence homologies are less than 30%, all th invariant residues found in animal lectin carbohydrate-recognition domains are conserved (Drickamer, J. Biol. Chem.

263:9557 (1988)). The lectin domain includes amino acid residues 42-170 given in Fig. 2.

The next domain of 36 amino acids, at residues 171-206 shown in Fig. 2, is homologous (36-39%) with epidermal growth factor (EGF) (Gregory, Nature 257:325 (1975)) and the EGF-like repeat units found in Factor IX (Yoshitake et al., Biochem. 25:3736 (1985)) and fibroblast proteoglycan core protein (Krusius et al., supra) (Fig. 3B).

Immediately following these domains are two tandem domains of 62 amino acids each, shown as residues 207-269 and 270-331 of Fig. 2, that are homologous with the short consensus repeat unit (SCR) that comprises the IL-2 receptor (Leonard et al., Nature 311:626 (1984)), Factor XIII (Ichinose et al., Biochem. 25:4633 (1986)) and many C3/C4 binding proteins (Klickstein et al., J. Exp. Med. 165:1095 (1987); and Morley et al., EMBO J. 3:153 (1984)). In contrast with all of the previously described SCR that contain four conserved Cys residues, each of these two SCR possesses six Cys residues. The four conserved Cys residues found in all SCR are indicated in Fig. 3C by (*); the two additional conserved Cys residues found in LAM-1 are indicated by (+). Of the multiple SCR present in each of these proteins, the SCR with the highest homology to LAM-1 is diagrammed (Fig. 3C). A 15 amino acid spacer follows the SCR units, preceding the transmembrane domain.

The expression of LAM-1 mRNA by cell lines of lymphoid and non-lymphoid origin was examined. Northern blot analysis revealed that LAM-1 cDNA hybridized strongly to a 2.6 kb RNA species and weakly to a 1.7 kb RNA species in poly(A)+ RNA isolated from the B cell lines Raji, SB, Laz-509, and GK-5. However, RNA isolat d from two pre-B c ll lin s (Nalm-6, PB-697), three B cell lines (Namalwa, Daudi, BJAB), five T cell lines (CEM, Hut-78, HSB-2, Molt-

15, Molt-3), a myelomonocytic cell line (U937 and U937 cultured with LPS) and erythroleukemic K-562 cell line did not hybridize with LAM-1 cDNA, suggesting that expression of this gene was preferentially associated with B lymphocytes. Neutrophils expressed LAM-1 mRNA but had a relatively lower amount of transcript among total mRNA when compared with the Raji cell line or blood T lymphocytes. LAM-1 cDNA has also been used to transfer expression of LAM-1 to cells that do not express the gene.

Preparation of mAb specific for LAM-1

MATERIALS AND METHODS

mAb. Anti-LAM-1 mAb produced by a total of 18 different hybridomas were analyzed. The anti-LAM-1 mAb identified as anti-LAM1-1, anti-LAM1-2, anti-LAM1-3 and anti-TQ1 have been previously described (Tedder et al., J. Immunol. 144:532, 1990; Reinherz et al., J. Immunol. 128:463, 1982; Gatenby et al., J. Immunol. 129:1997, 1982; Spertini et al., Leukemia (in press), 1991). The new anti-LAM-1 mAb (Table 7) were generated by the fusion of NS-1 myeloma cells with spleen cells from BALB/c mice that were repeatedly immunized with cells of the mouse pre-B cell line 300.19 stably transfected with a LAM-1 cDNA, as described (Tedder et al., J. Immunol. 144:532, 1990). Each hybridoma was cloned twice and used to generate ascites fluid. The isotypes of the mAb were determined by the immunostaining of cells preincubated with each anti-LAM-1 mAb with FITC-conjugated antibodies specific for the individual mouse H chain isotypes (Southern Biotechnology Associates, Birmingham, AL). FITC-conjugated anti-LAM-1 and Leu-8 mAbs were produced as described (Kansas et al., J. Immunol. 142:3058, 1989). Phycoerythrin-labelled anti-TQ1 was from Coulter Immunology (Hialeah, FL).

Cell samples and immunofluorescence analysis. Mononuclear cells were isolated from human, rhesus monkey (*Macaca mulatta*), cotton-topped tamarin (*Saguinus oedipus*), dog (*Canis familiaris*), cat (*Felis catus*), sheep (*Ovis aries*) or rabbit (*Oryctolagus cuniculus*) blood by Ficoll-Hypaque density gradient centrifugation. Neutrophils were isolated from blood samples at 20°C by centrifugation for 20 min at 1000 x g on a cushion of Mono-Poly Resolving Medium (Flow Laboratories, McLean, VA) followed by lysis of the red blood cells with ice-cold hypotonic 0.2% (w/v) NaCl solution. Cells were obtained by protocols approved by the Human Protection Committee and the Animal Care and Use Committee of Dana-Farber Cancer Institute. Cells were kept at 4°C and examined immediately after isolation.

Indirect immunofluorescence analysis was carried out after washing the cells three times. The cells were then incubated for 20 min on ice with each mAb as ascites fluid diluted to the optimal concentration for immunostaining. After washing, the cells were treated for 20 min at 4°C with FITC-conjugated goat anti-mouse Ig antibodies (Tago, Burlingame, CA). Single color immuno-fluorescence analysis was performed on an Epics Profile flow cytometer (Coulter Electronics, Hialeah, FL) or a FACStar (Becton Dickinson, Mountain View, CA). Ten thousand cells were analyzed in each instance.

Tissue sections were isolated from thymus and mesenteric lymph nodes of rabbit, pig (*Sus scrofa*), goat (*Capra hircus*), rat (*Rattus norvegicus*), guinea pig (*Cavia porcellus*) and chicken (*Gallus domesticus*), and from rabbit appendix. These frozen sections were stained with a given anti-LAM-1 mAb at optimal concentrations, with subsequent development using immunohistological procedures as described (Mackay et al., J. Exp. Med. 167:1755, 1988).

PPME binding assays. FITC-labeled PPME (PPME-FITC), was used for staining peripheral blood lymphocytes as described (Spertini et al., Leukemia (in press) 1991; Yednock et al., J. Cell. Biol. 104:713, 1987). Briefly, cells were treated for 20 min on ice with each anti-LAM-1 mAb, washed and then incubated for 30 min on ice in PBS containing 3 μg/ml PPME-FITC. After fixation in PBS-formaldehyde (1% v/v), PPME-FITC binding was analyzed by flow cytometry. In each experiment, the percentage of PPME-FITC binding cells was less than 5% when the staining was carried out in PBS containing 5 mM EDTA. The live cell binding assay used to assess the binding of iodinated PPME by lymphocytes was carried out as described previously (Spertini et al., Nature 349:691, 1991).

Unlabeled PPME was a gift of Dr. Morey Slodki (Northern Regional Research Center, Peoria, IL) and was used to block anti-LAM-1 mAb binding to lymphocytes by first incubating the lymphocytes at 4°C in PBS containing 300 μg/ml PPME. After 45 min of incubation, the anti-LAM-1 mAbs were added to the cell suspension for 10 min. After washing, the lymphocytes were incubated for 20 min with FITC-labeled goat anti-mouse Ig antibodies, with subsequent analysis by flow cytometry.

Effect of anti-LAM-1 mAb on lymphocyte binding to HEV. The *in vitro* HEV binding assay was performed with frozen tissue sections of rat peripheral lymph nodes using the methods of Stamper and Woodruff (Stamper et al., J. Exp. Med. 144:828, 1976) and Butcher et al. (Butcher et al., J. Immunol. 134:2989, 1979) as described (Tedder et al., J. Immunol. 144:532, 1990). Briefly, lymphocyt s ($4x10^6$) were treat d with neuraminidase (0.005 U/ml) for 30 min at room temperature, th n incubated for 10 min on ic with th anti-

LAM-1 mAbs as ascites fluid diluted at 1:100 in RPMI 1640 containing 5% fetal calf serum. The anti-LAM1-3 and -4 mAb could be used at considerable dilution (1:5000) without a decrease in their ability to inhibit HEV binding. The cells, in a final volume of 100 µl, were then incubated under rotation (64 rpm) for 30 min at 4°C on four 12 µm frozen rat peripheral lymph node sections. After fixation overnight in PBS with 1% (w/v) glutaraldehyde, the number of HEVs per tissue section was determined and the number of lymphocytes adherent to HEV was quantitated.

Antibody cross-blocking experiments. Lymphocytes (5×10^5) were first incubated with 10-fold saturating concentrations of one anti-LAM-1 mAb as diluted ascites fluid for 20 min on ice followed by the addition of optimal concentrations of the second fluorochrome-labelled anti-LAM-1 mAb to be used for direct immunofluorescence analysis. After 20 min of further incubation, the cells were washed and mAb binding assessed immediately by flow cytometry, as described (Tedder et al., J. Immunol. 144:532, 1990). Optimal concentrations for each antibody were determined by indirect immunofluorescence analysis.

Domain mapping of epitopes defined by anti-LAM-1 mAb. As described in detail elsewhere (Kansas et al., J. Cell Biol. (in press) 1991), various domains of CD62 were substituted for those of LAM-1 to create chimeric selectins containing specific domains of LAM-1 with the remainder of the protein as CD62. In particular, cDNAs encoding fusion proteins containing the lectin domain, the lectin plus EGF domains, or the lectin, EGF plus SCR domains of LAM-1, with the remaind r as CD62, were created and subcl ned into the Ap^rM8 expression vect r (pr vided by Dr. Lloyd Klickstein, Center for Blood Research, Bost n, MA). These cDNAs were

expressed in COS cells to determine which LAM-1 domains were recognized by the anti-LAM-1 mAb in indirect immunofluorescence assays. The cells were examined by fluorescence microscopy, and only those cells exhibiting obvious membrane fluorescence were scored as positive.

RESULTS

Characterization of the anti-LAM-1 mAb. MAb-producing hybridomas were generated by the fusion of NS-1 myeloma cells with spleen cells from mice immunized with LAM-1 cDNA-transfected 300.19 cells (Tedder et al., J. Immunol. 144:532, 1990). Hybridoma cells producing mAb that were reactive with LAM-1 cDNA-transfected 300.19 cells, but not with CD20-transfected cells, were isolated. Thirteen of twenty new anti-LAM-1 mAb were characterized, of which eleven were IgG_1 and two were IgM (Table 7). Each of these mAb reacted specifically with COS cells, K562 erythroleukemia cells, and NALM-6 pre-B cells transfected with the LAM-1 cDNA (Tedder et al., J. Immunol. 144:532, 1990), but not with those cells untransfected or transfected with CD19 or CD20 cDNAs (Tedder et al., Proc. Natl. Acad. Sci. USA 85:208, 1988; Tedder et al., J. Immunol. 143:712, 1989). In addition, each of these mAb reacted with six human LAM-1+ lymphoblastoid cell lines, but were unreactive with four LAM-1-lymphoblastoid or myeloid cell lines. Therefore, these mAb definitively identify the protein encoded by the LAM-1 cDNA (Tedder et al., J. Exp. Med. 170:123, 1989).

The 13 new anti-LAM-1 mAb, along with anti-LAM1-1, anti-LAM1-2, anti-LAM1-3, and anti-TQ1 mAb, were used to further characterize LAM-1 structure and function. Indirect immunofluorescence staining of blood lymphocytes and neutr phils showed that the fluor scence intensity of

staining with the anti-LAM-1 mAb ranged from low staining (+) to bright staining (++++) (Fig. 9, Table 7). No mAb were detected that stained lymphocytes or neutrophils preferentially. The level of staining observed was characteristic of each mAb, and was not due to low or insufficient mAb concentrations, because mAb levels greater than saturation generated identical results; or to isotype differences, because 14 of the 17 antibodies were of the same Ig subclass (i.e. IgG_1).

Inhibition of Lymphocyte binding to HEV is blocked by some anti-LAM-1 mAb. The anti-LAM-1 mAb were tested for their ability to inhibit lymphocyte binding to HEV of rat peripheral lymph node sections in the *in vitro* frozen section assay (Stamper et al., J. Exp. Med. 144:828, 1976). The anti-LAM1-3, anti-LAM1-4 and anti-LAM1-6 mAb inhibited lymphocyte binding by 85 to 90% (Table 8). In contrast to our previous report (Tedder et al., J. Immunol. 144:532, 1990), the anti-LAM1-1 and anti-LAM1-2 mAb consistently inhibited binding to an intermediate degree (~65% and ~45%, respectively). Our previous inability to detect this inhibition may be secondary to the previous use of human lymph nodes as a source of HEV; the likely involvement of LFA-1 in binding to (presumably inflamed) human lymph nodes (Stamper et al., J. Exp. Med. 144:828, 1976), may have obscured the true effects of these mAb. The remaining 12 anti-LAM-1 mAb had little or no effect on lymphocyte binding to HEV under the conditions examined (Table 7). This lack of effect did not result from the presence of insufficient mAb for inhibition, since each mAb was used at a concentration 2 to 10 fold higher than that required for saturati n, as determined by immun fluorescence staining.

Modulation of PPME-FITC binding by anti-LAM-1 mAb. In order to correlate the effect of the anti-LAM-1 mAb on HEV binding with their ability to inhibit LAM-1/carbohydrate interactions, the effect of these mAb on the binding of PPME-FITC was examined. Two of the 17 anti-LAM-1 mAb used in this study, anti-LAM1-3 and anti-LAM1-4, completely blocked the binding of PPME-FITC to lymphocytes to the same extent as the treatment of lymphocytes with 5 mM EDTA Fig. 10. The anti-LAM1-2 and TQ1 mAb had a similar inhibitory effect. Surprisingly, the prior binding of anti-LAM1-1 or anti-LAM1-5 mAb to lymphocytes greatly enhanced PPME-FITC binding (Fig. 10) similar to the binding increase observed immediately following lymphocyte activation. Preincubation of lymphocytes with the anti-LAM1-3 mAb before treatment with enhancing mAb blocked both basal and enhanced PPME binding (data not shown). Binding of the nine remaining anti-LAM-1 mAb, including anti-LAM1-10, did not inhibit PPME-FITC binding, as shown for the anti-LAM1-6 and -10 mAb (Fig. 10).

The enhanced binding of PPME induced by the anti-LAM1-1 mAb was further quantitated using iodinated PPME in a live cell binding assay, as described (Spertini et al., Nature 349:691, 1991). Lymphocytes treated at 4°C with medium bound 4,587 ± 372 cpm of PPME while those previously treated with anti-LAM1-1 mAb bound 23,195 ± 340 cpm of PPME, a five fold increase. This increase in PPME binding is comparable to that observed following lymphocyte activation through the T cell receptor complex or CD2 (Spertini et al., Nature 349:691, 1991). PPME binding was inhibited by the prior binding of the anti-LAM-3 mAb giving only 1160± 104 and 1892 ± 217 cpm b und, respectively. Similarly, treatment with EDTA reduced PPME binding to 578 and 424 cpm, respectively. Thus, binding of mAb to certain pitopes of

LAM-1 can inhibit PPME binding while mAb binding to different epitopes can upregulate binding.

Epitope localization of the anti-LAM-1 mAb binding sites. The spatial relationships between the different mAb binding epitopes were examined by mAb cross-blocking analysis (Table 9). Anti-LAM1-3 binding inhibited the subsequent binding of anti-LAM1-4, anti-TQ1 and anti-LAM1-2, but not anti-LAM1 mAb. Leu-8 binding was partially or totally blocked by most of the other mAb with the exception of anti-TQ1, anti-LAM1-2, -6 and -14. Taken together, this suggests that the anti-LAM1-3 and -4 epitopes are close to, but distinct from, the Leu-8 epitope, and farther from the epitopes defined by the other mAb. Anti-LAM1-7 and -8 partially, and -9, -10, -11, -12 and -15, totally, inhibited anti-LAM1-1, -5 and Leu-8 binding, indicating that these epitopes are clustered closely together. Anti-LAM1-6 appeared unique in this analysis, and therefore defines a spatially distant determinant. Finally, anti-LAM1-14 binding partially inhibited binding of anti-LAM1-1 only. Therefore, while most mAb-defined epitopes appeared to be closely associated within regions involved in ligand binding, distinct mAb-binding epitopes could be distinguished.

Domain mapping of epitopes identified by anti-LAM-1 mab. To identify the structural domains that contain epitopes defined by the anti-LAM-1 mAb, COS cells were transfected with cDNAs encoding chimeric (LAM-1/CD62) selectins and stained with anti-LAM-1 mAb (Table 10). With the exception of anti-LAM-1, -5, -14 and -15, each mAb bound the fusion protein containing only the lectin domain of LAM-1, and hence recognized the lectin domain. In combination with the mAb crossblocking analysis described above, this indicates that multiple antigenic sites are present within

the lectin domain, and that only some of these epitopes are functionally important. The anti-LAM1-1, -5 an -15 mAb recognized the fusion protein containing the lectin plus EGF domains of LAM-1, but not the fusion protein containing the lectin domain or lectin plus SCR domains only. Therefore, these mAb recognize epitopes within the EGF domain, or epitopes that are derived from the lectin plus EGF domains. Anti-LAM1-14 recognized only the fusion protein containing the LAM-1 SCR domains, and therefore defines a SCR epitope.

Evolutionary conservation of functional LAM-1 epitopes. Lymphocytes from a number of animal species were analyzed to determine if the functionally-defined epitopes described above were well conserved. In addition, we hypothesized that further distinctions between these different epitopes, as defined on human leukocytes, could be identified by their differential expression on various animal species. Rhesus monkeys, tamarin, rabbit, dog, cat, sheep, goat, cow and pig mononuclear cells each exhibited conservation of some LAM-1 epitopes (Table 11). However, none of the anti-LAM-1 mAb were reactive with rat, guinea pig or chicken mononuclear cells. The epitope recognized by anti-LAM1-3 was the most widely conserved among the different animal species tested (Fig. 11, Table 11). Unexpectedly, rhesus monkey mononuclear cells expressed this epitope at low levels, and tamarin mononuclear cells were not stained by LAM1-3, in contrast to animal species more phylogenetically distant. While the epitope identified by the anti-LAM1-4 mAb appears identical with the LAM1-3 epitope in all previous assays, its pattern of reactivity on animals was quite distinct. Similarly, within each group of mAb which previously were indistinguishable with respect to th ir functional, domain mapping and serological pr fil , differences in which animal species wer recogniz d were

detected (Table 11). The exceptions to this were the epitopes identified by the anti-LAM1-8 and -9 mAb and the epitopes identified by the anti-LAM1-10, -11 and -12 mAb, which recognized LAM-1 from the same species.

The finding that human and mouse lymphocytes bind PPME was extended to the study of tamarin, dog and rabbit LAM-1$^+$ lymphocytes (Fig. 12). PPME binding to lymphocytes from each of these species was completely inhibited by 5 mM EDTA. In addition, preincubation of cells from each of these three species with anti-LAM1-3 or -4 mAb diminished, albeit incompletely, PPME-FITC binding (Fig. 12). Finally, in two experiments, treatment of rhesus monkey blood mononuclear cells with anti-LAM1-3 inhibited 53% and 88% of lymphocyte binding to HEV, while treatment of dog mononuclear cells with anti-LAM1-3 inhibited 52% of the HEV binding. Thus, the functional and serologic properties of LAM-1 appear to be well conserved in diverse and phylogenetically disparate groups of mammals.

DISCUSSION

In this study, the LAM-1 epitopes which mediate or regulate HEV- and PPME- binding were characterized using a large panel of newly developed mAb (Table 7). In addition, the physical proximites of the functional regions defined by these mAb were identified, indicating that LAM-1 can be divided into a number of overlapping regions associated with distinct functional properties. Each mAb reacted with leukocytes with characteristic levels of immunofluorescence staining (Fig. 9, Table 7), suggesting that there may be heterogeneity in expression of LAM-1 epitopes. Thus, many of the anti-LAM-1 mAb may resemble the FMC46 mAb that identifies a cell protrusion-associated epitope of LAM-1. These mAb were also used to examine structural differences

between LAM-1 of lymphocytes and neutrophils. Both lymphocytes and neutrophils express a single species of LAM-1 protein, but the Mr of cell-surface LAM-1 on lymphocytes is 74,000 and that of neutrophil LAM-1 is 90-100,000. However, all of the anti-LAM-1 mAb reacted with both lymphocytes and neutrophils and each of the mAb stained neutrophils and lymphocytes with the same fluorescence intensity (Fig. 9, Table 7). Because each of the functional domains identified by the different mAb were conserved between leukocyte types, the structural differences between LAM-1 isoforms may only play a minor role in the regulation of leukocyte trafficking.

A study of the evolutionary conservation of LAM-1 epitopes expressed among different animals species indicated that at least twelve of the seventeen mAb used in this study identified unique LAM-1 epitopes (Table 7-11). The anti-LAM1-8, and -9 mAb may identify the same or related epitopes, while the anti-LAM1-10, -11 and -12 mAb were identical to each other in all respects (Table 7 and 11). The distinct functional properties of each epitope allows us to further divide LAM-1 into overlapping functional regions, with additional regions that are functionally silent. A model which summarizes the functional and serologic relationships of the LAM-1 epitopes identified in this report is presented in Fig. 13. The data also demonstrate that at least a portion of the EGF-like and SCR domains are accessible to antibodies and perhaps ligands as well. Importantly, comparison of the functional data with the serologic and domain mapping data reveals a close correlation.

On functionally dominant region of LAM-1 was defin d by the binding of two mAb, anti-LAM1-3 and anti-LAM1-4, which strongly inhibited the ability of lymphocytes

to bind both HEV and PPME. The DREG-56 mAb also fits into this functionally defined group of mAb (Kishimoto et al., Proc. Natl. Acad. Sci. USA 87:2244, 1990). The anti-LAM1-3 and -4 mAb bind the lectin domain of LAM-1 only (Table 10), and each completely blocked binding of the other (Table 9). The anti-LAM1-3 and anti-LAM1-4 mAb also completely block neutrophil binding of PPME. Two additional mAb, anti-LAM1-2 and TQ-1, also blocked PPME binding (Fig. 10). However, these mAb were less efficient at blocking lymphocyte binding to HEV (Table 8) suggesting that they may be more distant from the ligand binding site than the LAM1-3 and -4 epitopes. These mAb collectively define one functional group of LAM-1 epitopes and confirm previous work demonstrating a close correlation between the lectin activity of LAM-1 (and MEL-14) and the HEV binding ability of leukocytes (Yednock et al., J. Cell. Biol. 104713, 1987; Yednock et al., J. Cell. Biol. 104:725, 1987; Stoolman et al., J. Cell. Biol. 99:1535, 1984; Rosen et al., J. Immunol. 142:1895, 1989; Stoolman et al., J. Clin. Invest. 84:1196, 1989; Stoolman et al., Blood 70:1842, 1987).

Activation of lymphocytes through CD2 or CD3, or activation of neutrophils with TNF-α or GM-CSF, induces a rapid and transient increase in the affinity of LAM-1 for its ligand. An interesting and unexpected result of the current studies was that binding of the anti-LAM1-1 and -5 mAb resulted in an identical increase in PPME binding by LAM-1 (Fig. 10). Crossblocking studies indicated that these mAb bind to the same or overlapping regions, distinct from those defined by the anti-LAM1-3 and -4 mAb. Interestingly, domain mapping studies demonstrated that these epitopes are in, r require the presence of, the LAM-1 EGF-like domain. However, nly the anti-LAM1-1 mAb inhibited lymphocyte binding to HEV. The anti-LAM1-15 mAb, which blocks the

binding of both LAM1-1 and -5, also defines an epitopes within the EGF domain, but failed to affect the binding of PPME or HEV. Since the increase in PPME binding after binding of LAM1-1 or -5 occurred rapidly at 4°C, it is likely that mAb binding to LAM-1 in this region induces a conformational change in the molecule that results in an increased functional activity of the receptor for PPME. Therefore, it is possible that mAb binding to LAM-1 at these sites mimics a natural event or a component of the LAM-1 ligand, and that the region of the protein identified by LAM1-1 and -5 mAb serves a critical role in the regulation of receptor binding to ligand.

The epitope within the lectin domain identified by the anti-LAM106 mAb was also involved in ligand binding, but was distinct from the PPME binding site since this antibody has the ability to block HEV binding without modulating PPME binding. Crossblocking studies indicate that the anti-LAM1-6 mAb binds a region that is spatially separated from the site defined by the anti-LAM1-3 mAb. However this domain is close to the region defined by the anti-LAM1-1 mAb since these two mAb crossblock each other. The anti-LAM1-6 and anti-LAM101 binding sites are distinct since the former mAb binds the lectin domain of LAM-1 whereas the latter identifies the EGF-like domain. These results further demonstrate that the mAb approach to studying the conservation of functional epitopes on LAM-1 has many advantages over molecular genetic analysis of the primary structure, since the highly organized and folded nature of the LAM-1 molecule does not readily allow conclusions based on lin ar sequences and th crystal structure of LAM-1 has yet to be determined.

Human lymphocytes bind HEV of human or rodent peripheral lympho nodes with a similar specificity,

suggesting that the ligand for both murine and human lymphocytes is well conserved through recent mammalian evolution (Stoolman et al., Blood 70:1842, 1987; Stamper et al., J. Exp. Med. 144:828, 1976; Butcher et al., J. Immunol. 134:2989, 1979; Butcher et al., Nature 280:496, 1979; Wu et al., J. Cell. Biol. 107:1845). The demonstration that dog, tamarin and rabbit lymphocytes bind PPME in a calcium-dependent fashion (Fig. 12), in combination with the observation that dog and rhesus monkey lymphocytes utilized LAM-1 to bind to rodent HEV, confirms and extends this observation. These findings also indicate that the homing receptor expressed on leukocytes from these species involves a C-type lectin activity (Drickamer, J. Biol. Chem. 263:9557, 1988). Consistent with this, each mammal species (excluding rodents) tested expressed one or more of the LAM-1 epitopes (Table 11). The LAM1-3 epitope was the most broadly conserved among the animal species examined, further suggesting a critical function for this region in leukocyte migration. The reactivity of each mAb with numerous animal species also indicated that while several mAb were reactive with functionally identical regions of human LAM-1, most mAb identified unique epitopes. It is likely that subtle amino acid changes in LAM-1 between species accounts for the differences between reactivities of the anti-LAM-1 mAb. Thus these findings provide a mechanistic explanation for the conservation of receptor function, in that the regions of the molecule critical for adhesion were best conserved. Obtaining the primary amino acid sequences from LAM-1 cDNAs isolated from each species in the future will also be informative, but interpretation of sequence information in r gards to ligand binding sites and functional epitop s of the molecul will r quire the exact kn wledge of the thre dimensional structure of LAM-1.

Applicant's pending application Serial No. 07/313,109 describes a human cDNA sequence encoding the lymphocyte-assocated cell surface protein LAM-1, hereafter redesignated the leukocyte-adhesion molecule-1 (LAM-1), which contains domains homologous with binding domains of animal lectins, growth factors, and C3/C4 binding proteins, and the LAM-1 protein encoded by the cDNA sequence. Antagonists to LAM-1 were used in a method of treating a human patient suffering from a lymphocyte-mobilizing condition which involves administering a therapeutic amount of the antagonist in a non-toxic pharmaceutical carrier.

Normal leukocytes have the ability to leave the circulation and localize in specific lymphoid organs or inflammatory sites through interactions between cell-surface receptors and ligands on endothelial cells [references 1-3, see Glossary]. The Leukocyte-Adhesion Molecule-1 (LAM-1) contains an amino-terminal, lectin-like domain which may interact with specific glyco-conjugates expressed on high endothelial venules (HEV) of peripheral LN (lymph nodes) and activated endothelium [3-5]. LAM-1 is expressed by human peripheral lymphocytes, neutrophils, eosinophils, monocytes and hematopoietic progenitor cells [5-8]. LAM-1 is expressed by the majority of circulating lymphocytes and memory T cells, but is lost following several days of mitogen stimulation [5,9,10]. In contrast, LAM-1 is shed from the cell surface within minutes of exposure of lymphocytes and neutrophils to PMA [5,6,11]. Both lymphocytes and neutrophils express single LAM-1 protein product, but the molecular weight (Mr) of cell-surface LAM-1

on lymphocytes is 74,000 and that f neutrophils is 90,000-100,000 [6,9,12].

The specific adhesion of some tumor cells to the capillary endothelium and the existence of organ specific metastasis suggest that interactions between tumor cells and normal tissues influence tumor localization [13-15]. Although the molecules that mediate these events in malignant cells have not been completely described, many cell surface molecules involved in the adhesion and migration of normal leukocytes may be involved in the dissemination of hematopoietic malignant cells [1-3]. The mLHR has been implicated in the dissemination of lymphomas [14-16], and a calcium-dependent phosphomannosyl-binding site on human malignant lymphoblastoid cell lines mediates peripheral LN HEV binding [17]. In the invention described herein, the structure, function and regulation of LAM-1 expression was examined on normal lymphocytes and compared to LAM-1 of malignant leukocytes.

The LAM-1 molecule is a member of a new family of cellular adhesion/homing molecules that contain a lectin-like domain at their amino-terminal end followed by an epidermal growth factor-like domain and short consensus repeat units like those found in C3/C4 binding proteins. In J. Exp. Med., 170: 123-133 (1989) [4] and co-pending Application Serial No. 07/313,109, T.F. Tedder et al. report the isolation and chromosomal localization of cDNAs encoding the novel cell surface molecule LAM-1. In Eur. J. Immunol., 20: 1351-1355 (1990), T.F. T dd r t al. r p rted that human antig n-sp cifi mem ry T cells express th LAM-1 n cessary for lymphocyte recirculation. In J. Bi logical Chemistry,

265: 7760-7677 (1990), Ord t al. (und r the auspi es of T.F. Tedder) reported the stru ture f the gene encoding the LAM-1 of lymphocytes and neutrophils. In J. Immunology, 144: 532-542 (1990) [5], T.F. Tedder et al. described two monoclonal antibodies, LAM1-1 and LAM1-2, that react with LAM-1. [Note: As used herein, LAM-1 refers to the leukpocyte-adhesion molecule-1 itself and "LAM1-X" refers to an antibody x which binds to an epitope of LAM-1.)

The monoclonal antibodies LAM1-1 and LAM1-2 were found to be reactive with the majority of blood lymphocytes, NK (Natural Killer) cells, neutrophils, monocytes and hematopoeitic progenitor cells. Binding of LAM-1 may participate in the process of leukocyte extravasation into lymphoid organs or sites of acute inflammation with subsequent loss of LAM-1 from the cell surface. LAM-1 is also recognized by the TQ1 and Leu-8 monoclonal antibodies that have been previously identified.

The loss of LAM-1 expression after leukocyte activation in vivo, with the concomitant increase in expression of CD2, CD18, CD11a or CD11b may result in significant and dramatic increases in migration and ability to recognize endothelial cell surfaces. Of significance is the fact that patients with AIDs have diminished expression of LAM-1 on their T and B cells. This may also occur in other immunological syndromes. Therefore, alterations in LAM-1 expression by neutrophils are significant because the mLHR is involved in n utrophil migration into sites of acute inflammation. LAM-1, in c njuncti n with ther s l ctins and r c ptors, is involv d in th xtravasati n of m st leukocyt s. The xpression of LAM-1 by diff r nt leukocyt s sub-popul ti ns thus

plays a key role in determining the characteristics and magnitude of local immune responses [5].

The present invention relates to the production of a new antibody to LAM-1. The new monoclonal antibody, anti-LAM1-3, is useful in radioisotope or immunofluorescent assays for the detection of LAM-1. For example, identifying species which have or do not have LAM-1. The antibody is further useful for separating cells expressing LAM-1 from cells not expressing LAM-1 or visa versa. Furthermore, this monoclonal antibody also completely blocks leukocyte attachment to HEV or endothelium.

Neutrophil-mediated inflammation is involved in a number of human clinical manifestations, including the adult respiratory distress syndrome, multi-organ failure and reperfusion injury. One way of inhibiting this type of inflammatory response would be to block competitively the adhesive interactions between neutrophils and the endothelium adjacent to the inflamed region. Anti-LAM1-3 reacts with LAM-1 on many animal species, but does not bind the mLHR. Anti-LAM1-3 blocks completely lymphocytic traffic to lymph nodes and extravasation of neutrophils from blood to inflammatory sites. The administration of soluble forms of anti-LAM1-3 could be clinically effective for the inhibition of neutrophil-mediated inflammation. Anti-LAM1-3 also blocks lymphocyte adhesion to human HEV and activated endothelium. Therefore, it is likely that the use of anti-LAM1-3 will block lymphocyte entry into sites of inflammation or tissue injury. Such activity will be useful for preventing kidney or other organ transplant rejection which is mediated by lymphocytes.

It is also within the scope of the invention to prepare chimerized monoclonal antibodies from the mouse antibodies. Antibodies are Y-shaped molecules consisting of two long "heavy" chains which define the stem and arms of the Y and two short "light" chains which are attached to the outside of the arms. The amino-terminal ends of the arms of the antibody molecule contain the variable regions of the antibody. The variable regions are specific for a particular antigen. The stem of the molecule is the "constant" region which ends in a carboxylate function (COO^-) and remains the same from molecule to molecule in antibodies of the same isotype in the same species.

The constant region of the mouse antibody has been found to be the primary source human immune reactions to mouse monoclonal antibodies. Using standard genetic engineering techniques, mouse variable regions have been fused to human constant regions to generate "chimeric" (from chimera or chimaera, a monster of Greek mythology which had a lion's head, a goat's body and a serpent's tail) antibodies. These chimeric antibodies thus possess regions of different genetic origin and have been found to have a lower tenency to produce allergic reactions.

STATEMENT OF DEPOSIT

A hybrid cell line which produces the anti-LAM-1 monoclonal antibody anti-LAM1-3 embodying this invention was been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on June 12, 1991 and is assigned A.T.C.C. Deposit No. HB 10771.

DETAILED DESCRIPTION OF THE INVENTION

Glossary

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Definitions and Abbreviations

Selectins = a recently described family of cellular adhesion/homing receptor molecules identified by cDNA cloning. Members of this family include the leukocyt adh sion molecul -1 (LAM-1) which is the human h m l g of the mous lymphocyte homing r ceptor (mLHR), the human granul -membrane

protein (GMP-140, PADGEM, CD62) whi h is expressed on activated plat lets and endothelial cells, and the human endothelial leukocyte adhesion molecule-1 (ELAM-1) expressed on activated endothelial cells. The name "selectins" has been suggested for this family because of the presence of the lectin domain and their role in selective cell trafficking.

LN = lymph node

PMA = phorbol 12-myristate 13-acetate

PKC = protein kinase C

LAM-1 = leukocyte adhesion molecule-1

CLL = chronic lymphocytic leukemia

NHL = non-Hodgkin's lymphoma

PPME = poly-phosphomonoester from the yeast HANSENULA cell wall

AML = adult myelogenous leukemia

CML = chronic myelogenous leukemia

PBMC = peripheral blood mononuclear cells

BM = bone marrow

RPMI Medium = commercial product available from Gibco, Walkersville, Maryland.

FSC = follicular small cleaved cell lymphoma

CSF = colony stimulating factor

DSC = diffuse small cleaved cell lymphoma

FITC = fluorescein isothiocyanate

LPS = lipopolysaccharide

kb = kilobase

LAM-1 expressi n was examined n normal and ne plasti leukocytes to further understand the mechanisms that regulate leukocyte migration. The immunoprecipitation of a large fragment of LAM-1 of molecular weight 69,000 from the supernatant liquid of normal lymphocytes cultured with PMA demonstrated that LAM-1 can rapidly be cleaved from the cell surface (Fig. 4). That the LAM-1 expression is down-modulated by shedding rather than by internalization suggests that a PMA-sensitive regulatory pathway which is distinct from that which regulates down-modulation of most other surface molecules, controls the expression of LAM-1. This regulatory pathway may specifically involve the activation of PKC (Table 3). The presence of the soluble isoform of LAM-1 in the supernatant fluid of lymphocytes cultured without stimulation (Fig. 4) suggests that LAM-1 may also be continuously shed at a slow rate with its expression kept constant by the continuous synthesis of new receptors. Although the mechanism of shedding is unknown, enzymatic cleavage of the cell-surface receptor may result from the specific activation of a membrane bound protease. This is a likely method since a soluble protease secreted by activated leukocytes was not detected in this work. Alternatively, activation-induced changes in the comformation of the LAM-1 protein may expose nascent sites on LAM-1 that are then susceptible to cleavage by soluble proteases. Nonetheless, the finding that cell lines transfected with LAM-1 cDNAs rapidly modulat LAM-1 expression after PMA xposure (Fig. 8) sugg sts that the protease whi h cleav s LAM-1 is ubiquit us in distribution. The down-modulati n of LAM-1 by

shedding is similar to that of mLHR [33-34]. This is consistant with prior observations that the cell surface expression of LAM-1 is rapidly down-regulated upon activation [5,6,11]. LAM-1 removal from the cell surface may thus be necessary for the detachment of leukocytes from the endothelium so as to allow for their subsequent migration into tissues.

LAM-1 was most frequently expressed by CLL cells among the various hematologic malignancies studied (Table 1, Fig. 5). These results extend previous studies of LAM-1 expression to TQ1 and Leu-8 using CLL and NHL cells [35-37]. Since the expression of LAM-1 was somewhat restricted among hematologic malignancies, the expression, or absence of expression, may have a major impact on the trafficking of leukemic cells and the dissemination of NHL. Immunoprecipitation of LAM-1 from CLL cells showed that it resembled the Mr 74,000 isoform of the glycoprotein expressed by normal lymphocytes (Fig. 6). In addition, LAM-1 expressed by malignant cells was functional since LAM-1 on normal lymphocytes and CLL cells were both able to bind HEV and PPME (Table 2, Fig. 7). Both HEV and PPME binding was mediated by LAM-1 since the new monoclonal antibody, anti-LAM1-3, was able to completely block all HEV and PPME binding.

The level of HEV binding was also proportional to the quantity of LAM-1 expressed, and LAM-1 negative cells were unable to bind HEV and PPME. LAM-1 was also shed from the surface of CLL cells following PMA exposure (Fig. 8). However, the signalling pathway for shedding may be less active in some CLL cells since the time-course of LAM-1 shedding was slower than in normal lymphocytes. Malignant cells, therefore, express functional LAM-1 receptors that are indistinguishable from their normal counterparts on normal cells and the expression of LAM-1 by CLL cells correlated with the high tendency of these cells to localize into peripheral LN.

In contrast to LAM-1, CD44 expression was found to be consistently expressd at high levels among the leukemias and NHL examined, while the expression of other adhesion molecules CD11/CD18, CD54 and CD58 was variable (Table 1). Expression of CD44 did not correlate with the ability of cells to bind to HEV since LAM-1 negative CLL cells that expressed high levels of CD44 did not bind to HEV in frozen section assays (Table 2) similar to what was shown by one

research gr up using lymphoblast id cell lines [17]. CD44 c nstitutes a broadly distributed family of glycopr teins expressed on virtually all hematopoietic cells, fibroblasts, epidermal, glial and melanocytic origin cells [21,38]. Although CD44 was initially regarded as the human homing receptor equivalent of the mLHR [28,29], it may be more generally involved in cell-cell or cell-matrix binding as a receptor for hyaluronate [39]. Previous studies have also suggested that CD44 is involved in the dissemination of NHL [40]. During the work resulting in the present invention, however, no clear relationship could be inferred from the results of CD44 expression alone.

LAM-1 is expressed on most neutrophils, monocytes, normal myeloid progenitor cells and early erythroid precursors in BM (bone marrow) [6]. The co-expression of this homing receptor and other adhesion molecules may control the physiological retention (homing) of these cells in BM. The homing of intravenously transplanted hematopoietic stem cells is mediated by a recognition system with galactosyl and mannosyl specificities [41] which might also mimic the LAM-1 ligand [42]. In this regard, it is noted that AML and CML cells were found to lack expression of LAM-1. Unlike the situation with lymphoid tumors, this is in sharp distinction with the high level expression of LAM-1 on normal myeloid cells. The absence of LAM-1 expression on most AML and CML cells might favor the passage of these cells into the bloodstream. Although overnight culture of CML cells did not result in the expressi n of LAM-1 n th cell surfac , the ov rall lack of LAM-1 expr ssi n by th se ells indicat s that further investigations of th regulation of LAM-1 by l uk mic myeloid cells is warrant d.

There is growing evidence in mouse and man that the binding of lymphocytes to HEV of peripheral LN and the migration of normal leukocytes from blood into inflammatory lesions is controlled by several adhesion molecules whose expression is coordinately regulated [5,6,33,43]. It is likely that similar mechanisms will contribute to the spread of leukemias and lymphomas. In one murine study, the expression of functional receptors for HEV was shown to control the hematogenous dissemination of malignant lymphocyte populations to HEV bearing organs [16]. Lymphomas that bound well to HEV disseminated weakly vias the blood, ultimately involving all LN groups symmetrically. In contrast, gross involvement of LN by non-binding lymphomas was limited to nodes draining localized tumors which formed at the site of injection. These results suggested that the expression of functional receptors for HEV either controls the hematogenous dissemination of malignant lymphocyte populations to HEV-bearing organs, or is co-regulated with factors that determine metastatic behavior [16]. In humans, the expression of functional HEV binding molecules such as LAM-1, on CLL and low-grade lymphoma cells may also contribute to the wide-spread dissemination of these malignant cells to LN as occurs with normal lymphocytes.

Materials and methods

Cell Samples

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation of blood, bone marrow (BM) samples and single cell suspensions of LN. Cells were obtained by protocols approved by

the Human Protecti n Committee of th Dana-Farber Canc r Institute. Tum r type was lassified cc rding t conv n-tional morphological, cytological and immunophenotype criteria. Tumor cell lineage was determined by analysis of antigens (Ag) including surface and cytoplasmic immunoglobulin (Ig), HLA-DR Ag, CD1, CD2, CD3, CD4, CD5, CD6, CD8, CD9, CD10, CD11b, CD13, CD14, CD19, CD20 and CD33. Cells were examined immediately after isolation or were immediately cryopreserved and kept frozen in liquid nitrogen until used. The frequency of malignant cells was always greater than 90% in every sample examined.

Antibodies.

The anti-LAM-1 monoclonal antibodies anti-LAM1-1 and anti-LAM1-2 and the monoclonal antibody anti-TQ1 have been previously described [5,8]. The anti-LAM1-3 antibody (IgG1) of the claimed invention was generated by the fusion of NS-1 myeloma cells with spleen cells from Balb/c mice that were repeatedly immunized with cells of the mouse pre-B cell line 300.19 transfected with a LAM-1 cDNA as described [5]. The antibodies used in these studies included: 2F12 (CD11a) and 10F12 (CD18) [18] which were gifts from J. Ritz (Dana-Farber Cancer Inst., Boston MA); TS2/9 (CD58, anti-LFA-3) [19] and RR 1/1 (CD54, anti-ICAM-1) [20] which were gifts from T.A. Springer (Center for Blood Research, Boston, MA); 515 (CD44) [21] a gift from G.S. Kansas (Dana-Farber Cancer Inst.); and 904 (CD11b) [22].

Immunofluoresc nc analysis.

Indirect immunofluoresc nce analysis was perf rmed n viable cells isolated by Fic ll-Hypaque density gradi nt

centrifugation. The xpression f LAM-1, CD11a, CD11b, th β subunit of CD11 complex (CD18), CD44, CD54, and CD58 was examined by indirect immunofluorescence with flow cytometry analysis (Coulter Epics C, Coulter Electronics, Hialeah FL). Isotype-matched murine antibodies that were unreactive with human leukocytes were used as negative controls. Cells were incubated with each monoclonal antibody for 20 minutes on ice, washed, and treated with FITC-conjugated goat anti-mouse Ig reagents (Southern Biotechnology Associates, Birmingham, AL).

The ability of normal lymphocytes and B-CLL cells to bind the fluorescein derivative of PPME (fl-PPME) (a gift of S.D. Rosen, University of California, San Francisco, CA) was assessed by incubating cells for 30 minutes on ice with 100 µl of fl-PPME at 30 µg/ml in phosphate buffered saline (PBS). After washing twice, the binding of fl-PPME was examined by flow cytometry analysis as described [23,24].

HEV binding assay.

The <u>in vitro</u> HEV binding assay was performed using frozen tissue sections of human or rat peripheral LN using the methods of Stamper and Woodruff [25] and Butcher, et al. [26] as described [5]. Blocking of cell binding using the anti-LAM-1 monoclonal antibodies was carried out using fr shly cut-frozen rat lymph nod s ctions and the antibodies w re us d s ascites fluid at dilutions of 1:100.

Immun pre ipitati n analysis

Cells were washed twice, resusp nded in RPMI 1640 medium (Sigma, St. Louis, MO) at a concentration of 30 x 10^6 cells/ml) and treated for 40 minutes at room temperature with neuraminidase (0.1 U/ml, Calbiochem, La Jolla, CA) and then labelled by lactoperoxidase-catalyzed iodination. After washing, the cells were lysed in buffer containing 1% (v/v) NP-40 as described [27]. Cell lysates were precleared for 2 hours using 3 µl of murine ascites fluid (isotype matched antibody) and 25 µl of a 50% (v/v) suspension of Gammabind-G Agarose (Genex, Gaithersburg, MD). Cell lysates were precleared again overnight. Half of the precleared lysate was then incubated with 3 µl of anti-TQ1 ascites fluid, 3 µl of anti-LAM1-1 ascites fluid, and 50 µl of Gammabind-G with constant rotation at 4°C for 18 hours. The other half of the lysate was treated similarly using 3 µl of isotype-matched murine ascites fluid. Immunoprecipitates were washed and analyzed by SDS-PAGE. Molecular weights (Mr) were determined using standard molecular weight markers (BRL, Bethesda, MD).

In experiments designed to study LAM-1 shedding, LAM-1 was immunoprecipitated as described above from the supernatant fluid and the pellet of PBMC that had been cultured for 60 minutes at 37°C in RPMI 1640 medium alone or in RPMI medium containing PMA (100 ng/ml, Sigma, St. Louis, MO). In addition, expression of LAM-1 was assessed after incubation of the cells with PMA (10 nM for 30 minutes) following the pri r cultur f ths cells with sodium azid (Sigma) or the prot in kinas inhibit rs, 1-(5-Isoquinolinyl-sulfonyl)-2-m thylpip razin (H-7, Calbiochem) and staur sp rin (Sigma) for 30 minutes at 37 C.

Dis ussion f Figures 4-8.

Figure 4.

LAM-1 is shed from the cell surface into the culture medium. PBMC were surface iodinated and cultured for 60 minutes in medium or medium containing 100 ng/ml PMA. The supernatant fluid and cells were harvested and immunoprecipitated with anti-LAM-1 antibodies or an unreactive isotype matched control antibody (Cont.). Immunoprecipitated materials were electrophoresed on a 7.5% SDS acrylamide gel under reducing conditions followed by autoradiography. The migration of known molecular weight standards are shown in kilo-Daltons (kDa).

Figure 5.

The frequency of LAM-1 expression by malignant cells. Cells from 118 patients with various forms of hematopoietic malignancies were examined for surface LAM-1 expression using the anti-LAM1-1 monoclonal antibody in indirect immunofluorescence assays with flow cytometry analysis. In each instance, the background staining for each sample was determined using an unreactive isotype-matched monoclonal antibody and the level of background staining (usually less than 5%) was subtracted from the values shown. The horizontal bars represent the mean frequency of reactive cells. [Abbreviations: Pre-B, pre-B acute lymphoblastic leukemia; T-ALL, T cell acute lymphoblastic leukemia; B-CLL, B type chronic lymphocytic leukemia; FSC, follicular small cl av d c ll lymphoma; DSC, diffuse small cle ved cell lymphoma; DLC, diffuse large ell lymphoma; Burk., Burkitt's type lymphoma; M.M., multipl my l ma; AML, acute

myelogenous leukemia; CML, chronic myelogenous leukemia). The relative fluorescence staining intensity of the malignant cells is indicated where the positive population could be identified as a distinguishable peak from background fluorescence staining: ±, where a shoulder of positively stained cells was evident, +, where a separate peak of positive cells was identified with weak fluorescence; ++, a definite separate peak of fluorescence positive cells of moderate fluorescence; +++, a peak of fluorescence positive cells of the same intensity as normal blood lymphocytes. The tissue source of all malignant cells is also indicated.

Figure 6.

Anaylsis of LAM-1 immunoprecipitated from B-CLL cells. Detergent lysates of surface iodinated cells (45 x 10^6) were immunoprecipitated with the anti-TQ1 and anti-LAM1-1 antibodies (LAM-1) or an unreactive isotype-matched antibody control (Cont.). Immunoprecipitated materials were divided and analyzed under non-reducing and reducing conditions on a 12% SDS polyacrylamide gel followed by autoradiography. Molecular weights (kDa) were determined by the migration of known protein standards.

Figure 7.

Normal human lymphocytes and CLL cells are capable of binding PPME through LAM-1. Cells were examined for LAM-1 expression by indirect immunofluorescence analysis after treatment with the anti-LAM1-3 monoclonal antibody (dark line) or with an unreactive isotype matched antibody (thin line). Cells were also reacted with FITC-conjugated PPME after treatment with the anti-LAM1-3 antibody (thin line) or an unreactive control antibody (dark line). The

fluoresence intensity of 11 staining was analyzed by flow cytometry.

Figure 8.

Modulation of cell surface LAM-1 by malignant cells and cDNA transfected cells after PMA exposure. Cells transfected with LAM-1 cDNA, K562-LAM1 and 300.19-LAM1, and malignant cells that expressed LAM-1 were either cultured for 90 minutes in media or in media containing 10 ng/ml PMA. Following culture, the cells were examined for LAM-1 expression using the anti-LAM1-1 antibody in indirect immunofluorescence assays with flow cytometry analysis. Cells were also stained with an unreactive control antibody and the level of background staining was always less than 5%. The frequency of cells expressing LAM-1 is shown with the number of background staining cells subtracted.

Results

LAM-1 is released from the cell surface following PMA exposure.

Immunoprecipitation experiments were carried out to determine the fate of LAM-1 after modulation from the surface of PBMC exposed to PMA [5]. Surface iodinated cells were cultured for 60 minutes in RPMI medium alone or medium containing PMA. After culture, the supernatant fluid and cells were separated, and the cells were lysed with detergent. The cell lysate and supernatant fluid were immunoprecipitated with a combination of anti-LAM1-1 and anti-TQ1 antibodies that bind t diff r nt epitopes of LAM-1 [5], and tog ther are more effi i nt for immunoprecipitation. Th c lls wer also treated with n uraminidas prior to surfac

iodination since LAM-1 may be more readily immunoprecipitated after the removal of sialic acid residues. Treatment of cells with PMA resulted in a dramatic loss of immunoprecipitable LAM-1 from the cell surface with a concomitant increase in the level of LAM-1 precipitated from the supernatant fluid. Incubation of cells in medium without PMA also resulted in a small amount of LAM-1 being found in the supernatant fluid (Fig. 4). The molecular weight of LAM-1 precipitated from the supernatant fluid was slightly smaller (by about 5 kDa) than the species of LAM-1 found on the cell surface. Interestingly, the residual LAM-1 found on the cell surface of PMA-treated cells was most similar in molecular weight to that of the LAM-1 found in the supernatant fluid. The quantititive recovery of labeled LAM-1 from the supernatant fluid, in comparison to the amount immunoprecipitated from solubilized cells, demonstrates that a major portion of LAM-1 is shed from the cell surface and not internalized following PMA exposure.

Expression of adhesion molecules by malignant leukocytes.

The expression of LAM-1 and other cell surface molecules known to be involved in lymphocyte adhesion and migration was examined on malignant leukocytes from 118 patients by indirect immunofluorescence analysis. LAM-1 expression was most frequently demonstrated on CLL cells and among lymphomas classified as follicular (FSC) and diffuse small cleaved cell lymphoma (DSC) (Table 3). On the other hand, most acute myeloblastic leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelocytic leukemia (CML),

diffuse large cell lymphomas (DLC), Burkitt's lymphomas and multiple myelomas were LAM-1 negative. The level of cell surface LAM-1 expression was highest on CLL cells; and in 11 of 16 cases that expressed LAM-1, more than 50% of cells were LAM-1+ (FIG. 5). In general, the fluorescence intensity level of LAM-1 staining correlated with the frequency of LAM-1+ cells such that most malignant cell populations with less than 25% positive cells failed to express LAM-1 at easily detectable levels.

CLL cells rarely expressed adhesion molecules other than LAM-1, with the exception of CD44 (Table 3), which has also been associated with lymphocyte homing [28,29]. More than 90% of the cell samples were CD44+, consistent with its ubiquitous distribution on normal hematopoietic cells. Ninety-three percent of the leukemias and 84% of the B-NHL were positive for this antigen and generally greater than 60% of the cells were CD44+. In general, the expression of CD11a, CD11b, CD18, CD54, and CD58, was more heterogeneous (Table 3).

Incubating normal blood lymphocytes for about 8-16 hours at 4°C can result in complete loss of LAM-1 from the cell surface [30]. However, the cryopreservation of PBMC, LAM-1+ lymphoblastoid cell lines and freshly isolated LAM-1+ malignant cells did not appreciably alter the qualitative expression of LAM-1. A minor quantitative decrease in LAM-1 expression was observed that could be reversed by culturing the cells for about 8-16 hours in RPMI 1640 medium with 10% FCS (fetal calf serum). Consequently, 37 of the samples of malignant cells were re-examined following the above culture. This treatment, however, did not result in the

appearance f. LAM-1 n th ell surfaces or a significant in rease in th frequen y f LAM-1 expression in any case.

Structure of LAM-1 expressed by malignant cells.

Anti-LAM-1 antibodies were used to immunoprecipitate LAM-1 from CLL cells. LAM-1 migrated with a Mr of 68,000 under non-reducing conditions and at 73,000 after reduction (FIG. 6), similar to LAM-1 immunoprecipitated from normal lymphocytes (FIG. 4). Therefore, it appears that normal and malignant lymphocytes express the same cell-surface LAM-1 protein.

LAM-1 receptor function.

The relationship between LAM-1 expression and the ability of cells to bind to human peripheral LN HEV was examined using cells from normal circulating blood, three LAM-1 positive CLLs and one LAM-1 negative CLL. Cells were assessed for their ability to bind HEV of human peripheral LN using the frozen section assay of Stamper and Woodruff [25]. The LAM-1+ cells bound to HEV at levels which corresponded to the amount of LAM-1 expressed on their cell surface, while the LAM-1 CLL cells did not bind (Table 2). In contrast, CD44 expression was quite high on all of the cell samples examined and did not correlate with HEV adhesion. Additional studies examined the ability of anti-LAM-1 monoclonal antibody to block HEV binding. A new antibody, anti-LAM1-3, was able to specifically block 92 to 95% of normal lymphocyte and LAM-1+ CLL c ll binding (cells from Table 2) t rat peripheral LN HEV. In ntrast, the binding f a different antibody, anti-LAM1-10, r activ with a different

epit pe f LAM-1, had n det ctable ffect on HEV binding. (See Tabl 4). Theref re, the lev ls f LAM-1 expression correlated with the ability of cells to bind HEV and antibodies reactive with LAM-1 specifically blocked binding.

The ligand for the mLHR is mimicked by the mannose 6 phosphate-rich polysaccharide PPME [24]. Therefore, the ability of normal human lymphocytes and CLL cells to bind fluoresceinated PPME was examined to further characterize the functional capacity of human LAM-1. Both normal blood lymphocytes and LAM-1+ CLL cells were able to bind PPME, while LAM-1$^-$ CLL cells did not bind PPME (FIG. 7). The specificity of PPME binding to LAM-1 was verified by the ability of anti-LAM1-3 antibody to completely block PPME binding to the cells (FIG. 7).

Modulation of LAM-1 expresion.

The exposure of normal lymphocytes and neutrophils to phorbol esters induces a rapid disappearance of LAM-1 from the cell surface [5.6]. Therefore, modulation of LAM-1 expression after PMA stimulation was investigated on cells from 16 patients with CLL. After 90 minutes exposure to PMA, LAM-1 expression was completely lost in 4 cases, whereas it was only partially down-modulated in the 12 remaining cases. In 10 of these 12 cases, the down-modulation of LAM-1 expression was similar to that of RAJI cells cultured simultaneously under the same conditions (FIG. 8), while it was only minimal in two cases (B-CLL #1 and #2, Fig. 8). In cells fr m one pati nt with a FSC typ NHL, PMA induced an almost compl t m dulati n of LAM-1 expressi n after 90 minut s of stimulation (Fig. 8). In six

LAM-1+ CLL cell samples tested further. PMA exposure lead to the complete loss of LAM-1 expression after 180 to 360 minutes of culture with PMA with similar kinetics to those of RAJI cells treated simultaneously. These experiments were also carried out using cryo-preserved blood lymphocytes and RAJI cells as controls, with no appreciable affect on the ability of the cells to modulate LAM-1 expression after PMA exposure.

The down-modulation of LAM-1 expression was also investigated in LAM-1 cDNA transfected cells. The erythroleukemia cell line, K562, and the mouse pre-B cell line, 300.19, were transfected with LAM-1 cDNA as described [5], generating cells that express relatively high levels of cell surface LAM-1 (Fig. 8). In contrast to what was observed with RAJI cells and the majority of CLL cells, 90 minutes exposure of these cells to PMA induced an almost complete loss of LAM-1 from the cell surface.

The role of PKC in LAM-1 shedding was further assessed by culturing normal blood lymphocytes with protein kinase prior to their exposure to PMA. Treatment of cells with both H-7 [31] and staurosporine [32] inhibited shedding, albeit at different optimal molar concentrations (Table 1). In contrast, pretreatment of lymphocytes with sodium azide did not inhibit down-modulation of cell surface receptor. However, the shedding process required on-going metabolism since PMA treatment at 4°C did not induce detectable LAM-1 shedding (data not shown). Thus, PKC may regulate cell surface receptor expression through direct phosphorylation of LAM-1 which may signal for cleavage or through kinase regulation of proteas activity.

Shedding of LAM-1 does not result from the activation-induced secretion of a soluble protease. Neutrophils (10^7/ml) were activated with lineage-specific cytokines such as granulocyte/macrophage-CSF, to induce complete LAM-1 shedding [6]. The supernatant fluid of these cultures was harvested and used as culture medium for lymphocytes or LAM-1 cDNA transfected cells for 120 minutes at 37°C. This treatment did not induce detectable LAM-1 shedding from the surface of lymphocytes as assessed by flow cytometry analysis. In addition, the activation of neutrophils by lineage-specific stimuli in the presence of lymphocytes failed to induce detectable loss of lymphocyte LAM-1 while neutrophil shedding of LAM-1 was complete. Thus, it appears that a membrane anchored protease cleaves LAM-1 from the cell surface or that cellular activation is required for cleavage to occur.

LAM-1 expression on malignant leukocytes from tissue.

LAM-1 expression may be down-regulated during lymphocyte entry into tissues and this down-regulation is reversible in culture [5]. Malignant cells isolated from the highly infiltrated spleen of a CLL patient were found to express LAM-1 at a lower level than the CLL cells found in his peripheral blood (i.e 65% on blood cells and 25% on splenocytes). The percentage of LAM-1 positive spleen cells was comparable to that of the patients peripheral B-CLL cells stimulated for 180 minutes with PMA. This suggests that LAM-1 expression was decreased with entry of the CLL cells into the spleen as occurs with normal lymphocytes.

LN cells from patients with NHL (two patients with FSC, one with DLC and two with DSC) and leukemia BM cells (from

two patients with AML, n with ALL nd ne with CML) were incubated overnight at 37 C in RPMI 1640 m dium containing 10% FCS. Contrary to the results obtained under the same conditions with normal spleen cells [5], an increase in LAM-1 expression was not detected on the malignant cells after culture, suggesting that LAM-1 was not expressed constitutively on these cells.

Table 5, below, lists a number of the properties of the monoclonal antibodies anti-LAM1-1, -2 and -3. Lines 1, 2, and 3 give the name of the antibody, the isotype and the differences in staining intensity. These properties are not necesarily indicative of differences in epitope recognition.

Functional studies of the antibodies are given in lines 4-6. These results demonstrate that different parts of the LAM-1 molecule are recognized by the different monoclonal antibodies. Lymph nodes contain structures called high endothelial venules (HEV) which are utilized by lymphocytes to enter the lymph nodes (the site of immune responses) from the blood stream. Emigration of lymphocytes into the node has been shown to be mediated by adhesion molecules which allow the cells to stick to and then traverse the venule. This process has been studied by incubating isolated lymphocytes with lymph node tissue sections. When the sections are incubated with lymphocytes alone, the cells will adhere to HEV, and the number of adherent cells can be counted. Various monoclonal antibodies, including the LAM-1 antibodies, hav been us d to block this binding, Line 4 giv s the results of such studies for LAM1-1, -2 and -3.

The polysaccharid PPME mimi s the natural ligand f r the LAM-1 molecul . Since PPME can b dir ctly fluores-

ceinated, it is possibl t study th ff t f the vari us monoclonal antibodies n the interaction f LAM-1 and PPME. Line 5 details the results using cells which were first incubated with a LAM-1 monoclonal antibody, followed by treatment with PPME-FITC. Anti-TQ1, anti-LAM1-2 and anti-LAM1-3 blocked PPME binding and anti-LAM1-1 enhanced PPME binding. These results demonstrate the functional (and by extrapolation, the specificity) differences between the antibodies.

The results shown on line 6 were obtained by the reverse of the line 5 experiment; i.e., cells were first incubated with unlabelled PPME, followed by indirect immunofluorescence with the LAM-1 antibodies. As in line 5, differences in the effects of incubation with PPME on subsequent monoclonal antibody binding indicates that the various monoclonal antibodies recognize different epitopes of the LAM-1 molecule.

Lines 7-10 detail the results of studies in which the ability of a given monoclonal antibody to block the subsequent binding of other monoclonal antibodies was analyzed. Blocking of one antibody by another provides evidence that the two antibodies in question recognize epitopes which are identical or close together on the molecule. The results in Table 4 indicate that anti-LAM1-1 does not block anti-LAM1-3, indicating that their epitopes are different. Anti-LAM1-2, however, does block anti-LAM1-3, indicating that these pit pes r at least close to each other. There are dif- f r nces betwe n anti-LAM1-2 and anti-LAM1-3, h wever, be- c use of the difference respons they g nerat r garding Leu 8. Anti-LAM1-2 does not at ll block Leu 8, wh r as anti-

LAM1-3 strongly blocks it. Spe i s cross-r activity gives further indic ti ns f the difer n s which xist betwe n the antibodies and the epitopes that they identify.

Line 11 gives the results of the domain mapping regarding the monoclonal antibodies. The LAM-1 molecule contains three domains which are:

(a) a lectin-like domain (L);

(b) an epidermal growth factor-like domain (EGF); and

(c) a domain of short consesus repeats (SCR).

In order to determine which domain was recognized by each antibody, cDNAs were constructed which contained the information coding for:

(1) the whole LAM-1 molecule;

(2) the L, EGF and SCR domains from the LAM-1 molecule;

(3) the L domain from the LAM-1 plus EGF and SCR domains from the CD62 molecule of the same family of proteins;

(4) the L plus EGF domains from LAM-1 (SCR from CD62; and

(5) the L plus SCR domains from LAM-1 (EGF from CD62).

These cDNAs were transfected into cells which then produced the corresponding proteins. The pattern of reactivity of the various monoclonal antibodies was then determined as shown in Table 6, and the domain necessary for monoclonal antibody reactivity was assigned. For example, anti-LAM1-3 bound to cells expressing all the domains described with medium to very strong strength. Anti-LAM1-1, however, did not bind to c lls which contained LAM-1 (L + SCR) or LAM-1 (L) alone. Th pit pe which is r cognized by anti-LAM1-1 must, th r fore, b compos d f a sit within the EGF,

domain, or which contains part of the L and EGF domains, but not the SCR domain. LAM1-3, on the other hand, must only contain the LAM-1(L) domain. The two antibodies are, therefore, distinguishable.

The wide conservation of the LAM-1 epitopes expressed throughout recent mammalian evolution, in combination with the functional studies demonstrating a molecular component similar to LAM-1 in function and specificity, underscores the critical role of LAM-1 in the regulation of leukocyte migration in multiple animal species. Thus, LAM-1 may be the most functionally and structurally conserved leukocyte adhesion molecule found in mammals, considering that many do not function reciprocally between man and mouse. Whether the evolutionary basis for this high level of conservation is fundamental to the function of the receptor or results from the unique nature of a carbohydrate-based ligand with limited potential for divergence will have to be determined after identification and characterization of the true ligand(s).

Use

As leukocyte migration and infiltration into areas of tissue damage or injury or tissue transplant can cause or increase pathology, agents that impede these processes can be used for therapeutic treatment. Specifically, leukocyte-mediated inflammation is involved in a number of human clinical manifestations, including the adult respiratory distress syndrome, multi-organ failure and reperfusion injury. One way of inhibiting this type of inflammatory response would be to block competitively the adhesive interactions between leukocytes and the endothelium adjacent to the inflamed region. As LAM-1 mediates the migration and adhesion of blood leukocytes, treatment of a patient in shock, e.g., from a serious injury, with an antagonist to cell surface LAM-1 functi n (such as the monoclonal antibodies of th invention) can result in the reducti n of

leukocyte migration to a level manageable by the target endothelial cells.

In addition, agents developed to block receptor function can inhibit the metastasis and homing of malignant cells which express the LAM-1 receptor protein.

The therapeutic agents may be administered orally, parenterally, or topically by routine methods in pharmaceutically acceptable inert carrier substances. Optimal dosage and modes of administration can readily be determined by conventional protocols.

The normal regulation of the *lyam-1* gene (the name given to the human gene encoding LAM-1), as evidenced by the appearance and disappearance of the LAM-1 protein on the surface of a specific leukocyte subpopulation, can be monitored by use of the monoclonal antibodies of the invention, in order to test the effects of drugs or specific therapies that may alter gene expression.

What is claimed is:

Table 1. LAM-1 Sh dding Is Blocked By Prot in Kinease Inhibitors

		Without PMA[a] Treatment		With PMA Treatment	
Cells with		% Positive	MCF No.	% Positive	MCF No.
Medium		60	115	13	47
Stauro					
porine	1mM	57	91	54	95
	500μM	72	95	49	91
	100μM	51	88	51	92
	50μM	47	92	58	100
H-7	1mM	52	94	53	92
	500μM	67	102	32	80
	100μM	58	109	23	91
	50μM	61	106	11	61
NaN_3	1μM	59	113	20	50

a = The percentage of cells reactive with the anti-LAM1-1 monoclonal antibody was determined by indirect immunofluorescence analysis. The relative intensity of staining of the positive cells is indicated based on the mean fluorescence channel number (MFC No.) obtained with FACS analysis (256 chann ls, on a 3-d cad log sc l . C lls tr at d with an unr activ monocl nal antibody had 3% positive c lls with a MFC no. of 40.

Table 2. Binding Of Normal And Malignant Cells To HEV.

	LAM-1 Expression[a]		CD44 Expression		Cells Bound per HEV
Test	% Pos.	Intensity	% Pos.	Intensity	
1	70	+++	93	++++	2.12 ± 0.05
2	59	++	99	++++	1.02 ± 0.22
4	52	++	96	++++	1.02 ± 0.32
3	2	-	65	++++	0.18 ± 0.11
Normal PBL	85	+++	100	++++	4.64 ± 0.67

a = The percentage of cells with the anti-LAM1-1 and 515 monoclonal antibodies were determined by indirect immunofluorescence analysis. The relative intensity of staining of the positive cells is indicated based on a (-) being no reactivity and (++++) indicating the highest reactivity.

b = Values represent the mean number of cells (± SD) bound to each HEV. A total of 150 HEV were examined in each sample. The difference between the number of cells bound per HEV in LAM-1+ cases (1,2 and 4) and the LAM-1- case (3) was statistically significant ($P < 0.005$) using Student's t-t st statistic.

Tabl 3

EXPRESSION OF ADHESION MOLECULES BY MALIGNANT LEUKOCYTES

	No of cases expressing antigen / number examined[a] (mean % among positive cases)						
Diagnosis	LAM-1	CD44	CD11a	CD11b	CD18	CD54	CD58
Pre-B-ALL							
CD10+	4/15 (32)	10/12 (67)	1/8 (46)	0/15	1/12 (40)	4/13 (33)	6/13 (69)
CD10-	0/6	5/5 (80)	0/6	0/5	0/5	0/5	2/5 (55)
T-ALL	2/10 (31)	8/9 (67)	3/8 (59)	0/2	5/10 (49)	4/10 (46)	1/10 (62)
B-CLL	16/27 (55)	16/18 (76)	3/14 (45)	0/21	3/18 (32)	1/18 (31)	1/18 (43)
B-lymphoma							
FSC	6/12 (32)	9/11 (59)	8/9 (56)	0/12	9/11 (38)	5/11 (50)	2/11 (49)
DSC	2/4 (41)	4/4 (70)	1/4 (90)	0/3	1/4 (90)	3/4 (40)	1/4 (77)
DLC	1/6 (27)	6/6 (59)	5/6 (55)	0/6	4/4 (49)	3/6 (54)	2/6 (53)
Burkitt's	0/4	2/4 (52)	0/4	0/4	0/4	1/4 (72)	0/4
M. Myeloma	0/3	2/2 (84)	3/3 (53)		2/2 (37)	0/3	1/3 (52)
AML	2/19 (44)	16/16 (79)	6/11 (50)	5/19 (42)	11/16 (41)	1/16 (47)	9/16 (61)
CML	0/12	10/10 (61)	5/10 (43)	2/12 (48)	5/10 (39)	0/10	4/10 (70)

[a]Cases were considered positive for the Ag being examined if > 25% of the cells were positive.

Table 4. PPME and HEV binding are enhanced by lymphocyte activation.[1]

	Binding of [^{125}I]PPME (c.p.m. ± s.d.)			Cells Bound/HEV[2] (± s.d.)	
Stimulus	Medium	anti-LAM1-3	EDTA	Medium	anti-LAM1-3
Medium	4847±372	1160±104	578	1.4±0.2	0.2±0.2
anti-LAM1-10	6868±571	2103±905	2030	1.5±0.8	0.2±0.2
anti-CD2	17886±419	2863±689	965	2.4±0.9[4]	0.2±0.2
anti-CD3	19718±1294	1211±618	303	2.7±1.0[3]	0,2±0.2

1. Blood lymphocytes were isolated, incubated for 20 minutes at 4°C with the indicated antibodies, and anti-CD3 was crosslinked as shown by Spertini et al., Nature 349: 691-694 (1991) [See Spertini et al., Fig. 1]. After one wash, the cells were incubated with ^{125}I-labelled PPME 90.36 μg/ml, 2.2x10^5 c.p.m. per sample) at 4°C for 30 minutes. Anti-LAM1-3 was added 1 minute before the addition of the test antibody and during all incubations. The calcium-independent binding of [^{125}I]PPME was assessed in the presence of 5 mM EDTA. Cells were washed, resuspended in PBS-BSA and layered on a 750-μl cushion of 75% (v/v) calf serum. The cell pellet was isolated and bound [^{125}I]PPME assessed by γ (gamma) counting. These data are representative of those obtained in three experiments. Fluorescein-labelled PPME was iodinated by standard methods, the specific activity (2x10^4 c.p.m./ng) determined by self-displacement curve analysis, and the maximum binding capacity was 20%.

2. HEV binding was assessed using 12-μm freshly cut, frozen rat lymph node sections. The number of lymphocytes bound to HEV was counted on coded slides. Values are means ± standard deviation (s.d.) of four experiments, and the differences between control antibody-treated cells and anti-CD2 and anti-CD3 treated cells were significant.

3. $P<0.05$ using the paired Student's t-test.

4. $P<0.01$ using the paired Student's t-test

Table 5. Characteristic of Anti-LAM-1 Monoclonal Antibodies

Line		TQ1	LAM1-1	LAM1-2	LAM1-3
1	Antibody				
2	Isotype	G1	G1	M	G1
3	Fluoresc.[1]	++[7]	+++[7]	++	++++[7]
4	HEV Bind[2]	N	B	WB	B
5	PPME Bind[3]	B	E	B	B
6	PPME Blocks[4]	-[7]	+/-	+/-	-
	Ability to Block Binding of Labelled Monoclonal Antibody				
7	TQ-1[5]	+++	-	+++	+++
8	Leu 8[5]	-	++	-	+++
9	LAM1-1[5]	-	+++	-	-
10	LAM1-3[5]	+++	-	+++	+++
11	Domain Map[6]	L	L+EGF	L	L
	Species Cross Reactivity				
12	Rhesus	-	+++	-	+[1]
13	Tamarin	-	+++	++	--
14	Cow	ND	ND	ND	+++
15	Rabbit	+++	-	++	++++
16	Sheep	-	-	-	+++
17	Dog	-	-	-	+++
18	Cat	-	-	-	+++
19	Pig	ND	ND	ND	+
20	Goat	ND	ND	ND	+++
21	Epit p	C	A	B	D

1 = Fluorescence intensity, human lymphocytes and peripheral mononuclear cells. Fluorescence intensity of staining in indirect immunofluorescence assays, is given on a 4-(+) scale where (-) indicates no specific reactivity and (++++) indicates the highest level of activity.

2 = Monoclonal antibody blocking, High Endothelial Venule binding.

3 = Monoclonal antibody blocking, phosphomannan monoester fragments (PPME).

4 = PPME blocking monoclonal antibody.

5 = Ability to block binding of antibody.

6 = Domain mapped.

Abbreviations

Bind = Binding

B = Blocks

wB = weakly Blocks

E = Enhances

ND = Not Done

L = Lectin

EGF = Epidermal Growth Factor-like

SCR = Short Consensus Repeats

N = No effect

Table 6. Structural domains identified by the anti-LAM-1 monoclonal antibody reactive with fusion proteins containing LAM-1 domains[a].

Test mAb	Whole LAM-1	Lectin	Lectin EGF	Lectin EGF SCRs	Lectin SCRs
anti-LAM1-1	+++	-	+++	+++	-
anti-LAM1-2	ND	ND	ND	ND	ND
anti-LAM1-3	+++	+++	++	++++	++

a = Values represent the relative intensity of immunofluorescence staining of COS-7 cells transfected with the LAM-1 cDNA or recombinant cDNAs encoding the lectin, EGF-like, or two SCR domains of LAM-1 with the rest of the cDNAs encoding CD62.

SCR = a domain of short consensus repeats.

EGF = epidermal growth factor-like domain.

We claim:

Table 7

Properties of the anti-LAM-1 mAb used in this study

Name	mAb Isotype	Staining Intensity[a] Lympho.	Neutro.	HEV[b] Binding	Effect[c] on PPME
TQ1	G1	++	++	N	B
LAM1-1	G1	+++	+++	B	E
LAM1-2	M	++	++	wB	B
LAM1-3	G1	++++	++++	B	B
LAM1-4	G1	+++	+++	B	B
LAM1-5	G1	+++	+++	N	E
LAM1-6	G1	+	+	B	N
LAM1-7	G1	++	++	N	N
LAM1-8	G1	+++	+++	N	N
LAM1-9	G1	+++	+++	N	N
LAM1-10	G1	+++	+++	N	N
LAM1-11	G1	+++	+++	N	N
LAM1-12	G1	+++	+++	N	N
LAM1-13	M	+	+	N	N
LAM1-14	G1	+++	++	N	N
LAM1-15	G1	++	++	N	N
LAM1-16	M	+	+	N	N

[a] The reactivity of lymphocytes and neutrophils with the anti-LAM-1 mAb were determined by indirect immunofluorescence analysis with the relative intensity of fluorescence staining indicated on a - (no reactivity) to ++++ (highest reactivity) scale as in Fig. 1. Results are representative of those obtained in at least three experiments.

[b] The ability of each mAb to inhibit lymphocyte binding to rat HEV was assessed as in Table 8. B = Blocks binding, E = Enhances binding, N = No significant or detectable effect on binding, w = weak effect. Results are representative of those obtained in at least tw experiments.

[c] The ability of each mAb to inhibit PPME binding to human lymphocytes was as in Fig. 2. Results are representative f th se obtained in at least two experiments.

Table 8

Inhibition of human lymphocyte binding to rat HEV by anti-LAM-1 mAb[a]

mAb	# lymphocytes bound/HEV	# of experiments	% inhibition	Significance[b] (p value)
Medium	1.7 ± 0.9	8	-	-
LAM1-1	0.6 ± 0.4	6	65	< 0.025
LAM1-2	1.0 ± 0.2	2	42	<0.05
LAM1-3	0.3 ± 0.2	8	85	<0.005
LAM1-4	0.2 ± 0.1	3	90	< 0.025
LAM1-6	0.2 ± 0.1	3	90	< 0.025
LAM1-10	2.0 ± 0.9	4	0	none

[a] Human blood lymphocytes were assessed for their ability to bind to rat peripheral lymph node HEV as described in *Materials and Methods*. All HEV were counted regardless of lymphocyte binding with at least 150 HEV examined in each sample. The values represent the mean number of lymphocytes bound to each HEV (± SD) obtained in the number of experiments indicated.

[b] The statistical significance of the different values obtained with mAb treatment was compared with the values obtained with anti-LAM1-10 treatment using the Student's t test statistic in a paired analysis.

Tabl 9

Cross-Blocking Experiments with Anti-LAM-1 mAb[a]

	Ability of the test mAb to block the binding of labeled:				
Test mAb:	anti-TQ1	Leu-8	LAM1-1	LAM1-3	LAM1-5
TQ1	+++	-	-	+++	ND
LAM1-1	-	++	+++	-	+++
LAM1-2	+++	-	-	+++	ND
LAM1-3	+++	+++	-	+++	-
LAM1-4	+++	+++	-	+++	-
LAM1-5	++	++	+++	-	+++
LAM1-6	+++	-	+	-	-
LAM1-7	+++	++	++	-	++
LAM1-8	-	++	++	-	++
LAM1-9	-	+++	+++	-	+++
LAM1-10	-	+++	+++	-	+++
LAM1-11	-	+++	+++	-	+++
LAM1-12	-	+++	+++	-	+++
LAM1-14	-	-	++	-	-
LAM1-15	-	++	+++	-	+++

[a] Values represent the relative ability of the test mAb to block the binding of the indicated FITC-labeled antibodies: -, did not inhibit; +, partial inhibition; ++, significant but incomplete inhibition; +++, complete inhibition of mAb binding. These results are representative of those obtained in three experiments.

Table 10

Structural Domains Identified by the Anti-LAM-1 mAb

Test mAb:	Reactivity with fusion proteins containing LAM-1 domains:[a] Whole LAM-1	Lectin	Lectin EGF	Lectin EGF SCRs	Lectin SCRs
LAM1-1[b]	+++	-	+++	+++	-
LAM1-3[b]	+++	+++	++	++++	++
LAM1-4	+++	+++	++	++++	++
LAM1-5	+++	-	+++	++++	-
LAM1-6[b]	++	++	++	+++	++/+
LAM1-7	+++	+++	+++/++	+++	++/+
LAM1-8	++	+++	++	+++	+
LAM1-9	+++	+++	++	++++	++-
LAM1-10	+++	+++	++	++++	++/+
LAM1-11	+++	+++	+++	++++	++/+
LAM1-12	+++	+++	++	++++	++-
LAM1-14	+++	-	-	++++	++-
LAM1-15	+++	-	++	+++	-

[a] Values represent the relative intensity of immunofluorescence staining of COS-7 cells (- to +++ scale) transfected with the LAM-1 cDNA or chimeric cDNA encoding the lectin, EGF-like, or two SCR domains of LAM-1 with the rest of the cDNA encoding CD62. These results are representative of those obtained in at least three experiments.

[b] These results have been previously described in part (46).

Table 11

CONSERVATION OF LAM-1 EPITOPES AMONG DIFFERENT ANIMAL SPECIES

mAb	HUMAN[a]	RHESUS[a]	TAMARIN[a]	COW[a]	RABBIT[a,b]	SHEEP[a]	DOG[a]	CAT[a]	PIG[b]	GOAT[b]	RAT[a,b]	GUINEA PIG[b]	CHICKEN[b]
TQ1	+++	-	-	ND	+++	-	-	-	ND	ND	-	ND	ND
LAM1-1	+++	+++	+++	ND	-	-	-	-	ND	ND	-	ND	ND
LAM1-2	+++	-	++	ND	+ +	-	-	-	ND	ND	-	ND	ND
LAM1-3	++++	+	-	+++	++++	+++	+++	+++	+	+++	-	-	-
LAM1-4	+++	-	++	+ + +	+ + +	-	-	-	+ + +	ND	-	-	-
LAM1-5	+++	+++	+++	+ + +	+	+ + +	-	-	-	+ + +	-	-	-
LAM1-6	+++	+	+	-	+	-	-	-	-	ND	-	-	-
LAM1-7	+++	++	++	+ +	+ +	-	-	-	-	ND	-	-	-
LAM1-8	+++	++	+++	+++	-	++	-	-	-	ND	-	-	-
LAM1-9	+++	++	++	+++	-	+++	-	-	-	ND	-	-	-
LAM1-10	+++	++	++	+++	-	-	-	-	-	ND	-	-	-
LAM1-11	+++	+++	+++	+++	-	-	-	-	-	ND	-	-	-
LAM1-12	+++	+++	++	++	-	ND	-	-	-	ND	-	-	-
LAM1-13	±	-	-	-	+	+	-	-	-	ND	-	-	-
LAM1-14	+++	+	+	++	++	-	-	+	-	ND	-	-	-
LAM1-15	+++	++	++	+++	-	+++	-	-	-	+++	-	-	-
LAM1-16	+	-	+	-	-	+	-	-	-	ND	-	+	-

[a] Reactivity and relative fluorescence intensity of staining was determined by flow cytometry analysis of blood leukocytes. The scale of fluorescence intensities is as in Fig. 1 with - indicating no significant r activity. ND, not determined.

[b] Reactivity of staining was determined by immunohistology of thymus, lymph nodes and rabbit appendix.

The wide conservation of the LAM-1 epitopes expressed throughout recent mammalian evolution, in combination with the functional studies demonstrating a molecular component similar to LAM-1 in function and specificity, underscores the critical role of LAM-1 in the regulation of leukocyte migration in multiple animal species. Thus, LAM-1 may be the most functionally and structurally conserved leukocyte adhesion molecule found in mammals, considering that many do not function reciprocally between man and mouse. Whether the evolutionary basis for this high level of conservation is fundamental to the function of the receptor or results from the unique nature of a carbohydrate-based ligand with limited potential for divergence will have to be determined after identification and characterization of the true ligand(s).

Use

As leukocyte migration and infiltration into areas of tissue damage or injury or tissue transplant can cause or increase pathology, agents that impede these processes can be used for therapeutic treatment. Specifically, leukocyte-mediated inflammation is involved in a number of human clinical manifestations, including the adult respiratory distress syndrome, multi-organ failure and reperfusion injury. One way of inhibiting this type of inflammatory response would be to block competitively the adhesive interactions between leukocytes and the endothelium adjacent to the inflamed region. As LAM-1 mediates the migration and adhesion of blood leukocytes, treatment of a patient in shock, e.g., from a serious injury, with an antagonist to cell surface LAM-1 function (such as the m noclonal antibodies of the invention) can result in th reducti n f

leukocyte migration to a level manageable by the target endothelial cells.

In addition, agents developed to block receptor function can inhibit the metastasis and homing of malignant cells which express the LAM-1 receptor protein.

The therapeutic agents may be administered orally, parenterally, or topically by routine methods in pharmaceutically acceptable inert carrier substances. Optimal dosage and modes of administration can readily be determined by conventional protocols.

The normal regulation of the *lyam-1* gene (the name given to the human gene encoding LAM-1), as evidenced by the appearance and disappearance of the LAM-1 protein on the surface of a specific leukocyte subpopulation, can be monitored by use of the monoclonal antibodies of the invention, in order to test the effects of drugs or specific therapies that may alter gene expression.

What is claimed is:

CLAIMS

1. A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-4 monoclonal antibody.

2. The monoclonal antibody of claim 1, wherein said monoclonal antibody is anti-LAM1-4 monoclonal antibody.

3. A hybridoma cell which produces the monoclonal antibody of claim 1.

4. The hybridoma cell line deposited as ATTC #_____.

5. A method of identifying cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with a reagent comprising the monoclonal antibody of claim 1; and

determining which cells form an immune complex with said reagent.

6. A method of isolating cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with the monoclonal antibody of claim 1; and

separating those cells which have formed an immune complex with said monoclonal antibody, from those cells which have n t.

7. The method of claim 5, wherein said monoclonal antibody is anti-LAM1-4 monoclonal antibody.

8. A method of detecting leukocyte activation in an animal, said method comprising

obtaining a fluid sample from said animal;

contacting said sample with the monoclonal antibody of claim 1; and

detecting formation of immune complexes formed by said monoclonal antibody, said immune complexes indicating the presence of LAM-1 shed from activated leukocytes.

9. The method of claim 8, wherein said monoclonal antibody is anti-LAM1-4 monoclonal antibody.

10. A method of blocking leukocyte interactions with endothelium in an animal, said method comprising administering an effective amount of the monoclonal antibody or fragment thereof of claim 1 to said animal.

11. The method of claim 10, wherein said monoclonal antibody is anti-LAM1-4 monoclonal antibody.

12. The method of claim 10, wherein said animal is a human.

13. The method of claim 10, wherein said animal is suffering from inflammation.

14. The method of claim 10, wherein said animal is suffering from an autoimmune response.

15. The method of claim 10, wherein said animal is suffering from rejection of an organ or tissue transplant.

16. The method of claim 12, wherein said monoclonal antibody is a chimerized antibody having a variable region derived from anti-LAM1-4 monoclonal antibody and constant regions derived from a human antibody.

17. A method of determining the degree of expression of LAM-1 in a sample of leukocytes, said method comprising

contacting a sample of leukocytes with the antibody of claim 1, and

determining the level of immune complex formation in said sample, said level being indicative of the degree of expression of LAM-1 on said leukocytes.

18. A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-5 monoclonal antibody.

19. The monoclonal antibody of claim 18, wherein said monoclonal antibody is anti-LAM1-5 monoclonal antibody.

20. A hybridoma cell which produces the monoclonal antibody of claim 18.

21. The hybridoma cell line deposited as ATTC #_____.

22. A method of identifying cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspect d of expressing LAM-1;

contacting said cells with a reagent comprising the monoclonal antibody of claim 18; and

determining which cells form an immune complex with said reagent.

23. A method of isolating cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with the monoclonal antibody of claim 18; and

separating those cells which have formed an immune complex with said monoclonal antibody, from those cells which have not.

24. The method of claim 22, wherein said monoclonal antibody is anti-LAM1-5 monoclonal antibody.

25. A method of detecting leukocyte activation in an animal, said method comprising

obtaining a fluid sample from said animal;

contacting said sample with the monoclonal antibody of claim 18; and

detecting formation of immune complexes formed by said monoclonal antibody, said immune complexes indicating the presence of LAM-1 shed from activated leukocytes.

26. The method of claim 25, wherein said monoclonal antibody is anti-LAM1-5 monoclonal antibody.

27. A method of blocking leukocyte interactions with ndothelium in an animal, said method comprising

administering an effective amount of the monoclonal antibody or fragment thereof of claim 18 to said animal.

28. The method of claim 27, wherein said monoclonal antibody is anti-LAM1-5 monoclonal antibody.

29. The method of claim 27, wherein said animal is a human.

30. The method of claim 27, wherein said animal is suffering from inflammation.

31. The method of claim 27, wherein said animal is suffering from an autoimmune response.

32. The method of claim 27, wherein said animal is suffering from rejection of an organ or tissue transplant.

33. The method of claim 29, wherein said monoclonal antibody is a chimerized antibody having a variable region derived from anti-LAM1-5 monoclonal antibody and constant regions derived from a human antibody.

34. A method of determining the degree of expression of LAM-1 in a sample of leukocytes, said method comprising

contacting a sample of leukocytes with the antibody of claim 18, and

determining the level of immune complex formation in said sample, said level being indicative of the degree of expression of LAM-1 on said leukocytes.

35. A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-6 monoclonal antibody.

36. The monoclonal antibody of claim 35, wherein said monoclonal antibody is anti-LAM1-6 monoclonal antibody.

37. A hybridoma cell which produces the monoclonal antibody of claim 35.

38. The hybridoma cell line deposited as ATTC #_____.

39. A method of identifying cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with a reagent comprising the monoclonal antibody of claim 35; and

determining which cells form an immune complex with said reagent.

40. A method of isolating cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with the monoclonal antibody of claim 35; and

separating those cells which have formed an immune complex with said monoclonal antibody, from those cells which have not.

41. The method f claim 39, wher in said mon clonal antibody is anti-LAM1-6 monoclonal antibody.

42. A method of detecting leukocyte activation in an animal, said method comprising

obtaining a fluid sample from said animal;

contacting said sample with the monoclonal antibody of claim 35; and

detecting formation of immune complexes formed by said monoclonal antibody, said immune complexes indicating the presence of LAM-1 shed from activated leukocytes.

43. The method of claim 42, wherein said monoclonal antibody is anti-LAM1-6 monoclonal antibody.

44. A method of blocking leukocyte interactions with endothelium in an animal, said method comprising administering an effective amount of the monoclonal antibody or fragment thereof of claim 35 to said animal.

45. The method of claim 44, wherein said monoclonal antibody is anti-LAM1-6 monoclonal antibody.

46. The method of claim 44, wherein said animal is a human.

47. The method of claim 44, wherein said animal is suffering from inflammation.

48. The method of claim 44, wherein said animal is suffering from an autoimmune response.

49. The method f claim 44, wherein said animal is suffering from r jection of an organ or tissue transplant.

50. The method of claim 46, wherein said monoclonal antibody is a chimerized antibody having a variable region derived from anti-LAM1-6 monoclonal antibody and constant regions derived from a human antibody.

51. A method of determining the degree of expression of LAM-1 in a sample of leukocytes, said method comprising

contacting a sample of leukocytes with the antibody of claim 35, and

determining the level of immune complex formation in said sample, said level being indicative of the degree of expression of LAM-1 on said leukocytes.

52. A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-7 monoclonal antibody.

53. The monoclonal antibody of claim 52, wherein said monoclonal antibody is anti-LAM1-7 monoclonal antibody.

54. A hybridoma cell which produces the monoclonal antibody of claim 52.

55. The hybridoma cell line deposited as ATTC #_____.

56. A method of identifying cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with a reagent c mprising the monoclonal antibody of claim 52; and

determining which cells form an immune complex with said reagent.

57. A method of isolating cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with the monoclonal antibody of claim 52; and

separating those cells which have formed an immune complex with said monoclonal antibody, from those cells which have not.

58. The method of claim 56, wherein said monoclonal antibody is anti-LAM1-7 monoclonal antibody.

59. A method of detecting leukocyte activation in an animal, said method comprising

obtaining a fluid sample from said animal;

contacting said sample with the monoclonal antibody of claim 52; and

detecting formation of immune complexes formed by said monoclonal antibody, said immune complexes indicating the presence of LAM-1 shed from activated leukocytes.

60. The method of claim 59, wherein said monoclonal antibody is anti-LAM1-7 monoclonal antibody.

61. A method of blocking leukocyte interactions with ndothelium in an animal, said method comprising administering an effective amount of the monoclonal antibody or fragment thereof of claim 52 to said animal.

62. The method of claim 61, wherein said monoclonal antibody is anti-LAM1-7 monoclonal antibody.

63. The method of claim 61, wherein said animal is a human.

64. The method of claim 61, wherein said animal is suffering from inflammation.

65. The method of claim 61, wherein said animal is suffering from an autoimmune response.

66. The method of claim 61, wherein said animal is suffering from rejection of an organ or tissue transplant.

67. The method of claim 63, wherein said monoclonal antibody is a chimerized antibody having a variable region derived from anti-LAM1-7 monoclonal antibody and constant regions derived from a human antibody.

68. A method of determining the degree of expression of LAM-1 in a sample of leukocytes, said method comprising

contacting a sample of leukocytes with the antibody of claim 52, and

determining the level of immune complex formation in said sample, said level being indicative of the degree of expression of LAM-1 on said leukocytes.

69. A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-8 monoclonal antibody.

70. The monoclonal antibody of claim 69, wherein said monoclonal antibody is anti-LAM1-8 monoclonal antibody.

71. A hybridoma cell which produces the monoclonal antibody of claim 69.

72. The hybridoma cell line deposited as ATTC #_____.

73. A method of identifying cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with a reagent comprising the monoclonal antibody of claim 69; and

determining which cells form an immune complex with said reagent.

74. A method of isolating cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with the monoclonal antibody of claim 69; and

separating those cells which have formed an immune complex with said monoclonal antibody, from those cells which have not.

75. The method of claim 73, wherein said monoclonal antibody is anti-LAM1-8 monoclonal antibody.

76. A method of detecting leukocyte activation in an animal, said meth d comprising

obtaining a fluid sample from said animal;
contacting said sample with the monoclonal antibody of claim 69; and
detecting formation of immune complexes formed by said monoclonal antibody, said immune complexes indicating the presence of LAM-1 shed from activated leukocytes.

77. The method of claim 76, wherein said monoclonal antibody is anti-LAM1-8 monoclonal antibody.

78. A method of blocking leukocyte interactions with endothelium in an animal, said method comprising administering an effective amount of the monoclonal antibody or fragment thereof of claim 69 to said animal.

79. The method of claim 78, wherein said monoclonal antibody is anti-LAM1-8 monoclonal antibody.

80. The method of claim 78, wherein said animal is a human.

81. The method of claim 78, wherein said animal is suffering from inflammation.

82. The method of claim 78, wherein said animal is suffering from an autoimmune response.

83. The method of claim 78, wherein said animal is suffering from rejection of an organ or tissue transplant.

84. The method of claim 80, wherein said monoclonal antibody is a chimerized antibody having a variable region

derived from anti-LAM1-8 monoclonal antibody and constant regions derived from a human antibody.

85. A method of determining the degree of expression of LAM-1 in a sample of leukocytes, said method comprising

contacting a sample of leukocytes with the antibody of claim 69, and

determining the level of immune complex formation in said sample, said level being indicative of the degree of expression of LAM-1 on said leukocytes.

86. A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-9 monoclonal antibody.

87. The monoclonal antibody of claim 86, wherein said monoclonal antibody is anti-LAM1-9 monoclonal antibody.

88. A hybridoma cell which produces the monoclonal antibody of claim 86.

89. The hybridoma cell line deposited as ATTC #_____.

90. A method of identifying cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with a reagent comprising the monoclonal antibody of claim 86; and

determining which cells form an immune complex with said reagent.

91. A method of isolating cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with the monoclonal antibody of claim 86; and

separating those cells which have formed an immune complex with said monoclonal antibody, from those cells which have not.

92. The method of claim 90, wherein said monoclonal antibody is anti-LAM1-9 monoclonal antibody.

93. A method of detecting leukocyte activation in an animal, said method comprising

obtaining a fluid sample from said animal;

contacting said sample with the monoclonal antibody of claim 86; and

detecting formation of immune complexes formed by said monoclonal antibody, said immune complexes indicating the presence of LAM-1 shed from activated leukocytes.

94. The method of claim 93, wherein said monoclonal antibody is anti-LAM1-9 monoclonal antibody.

95. A method of blocking leukocyte interactions with endothelium in an animal, said method comprising administering an effective amount of the monoclonal antibody or fragment thereof of claim 86 to said animal.

96. The method of claim 95, wherein said monoclonal antibody is anti-LAM1-9 monoclonal antibody.

97. The method of claim 95, wherein said animal is a human.

98. The method of claim 95, wherein said animal is suffering from inflammation.

99. The method of claim 95, wherein said animal is suffering from an autoimmune response.

100. The method of claim 95, wherein said animal is suffering from rejection of an organ or tissue transplant.

101. The method of claim 95, wherein said monoclonal antibody is a chimerized antibody having a variable region derived from anti-LAM1-9 monoclonal antibody and constant regions derived from a human antibody.

102. A method of determining the degree of expression of LAM-1 in a sample of leukocytes, said method comprising

contacting a sample of leukocytes with the antibody of claim 86, and

determining the level of immune complex formation in said sample, said level being indicative of the degree of expression of LAM-1 on said leukocytes.

103. A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-10 monoclonal antibody.

104. The monoclonal antibody of claim 103, wherein said monoclonal antibody is anti-LAM1-10 monoclonal antibody.

105. A hybridoma cell which produces the monoclonal antibody of claim 103.

106. The hybridoma cell line deposited as ATTC #_____.

107. A method of identifying cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with a reagent comprising the monoclonal antibody of claim 103; and

determining which cells form an immune complex with said reagent.

108. A method of isolating cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with the monoclonal antibody of claim 103; and

separating those cells which have formed an immune complex with said monoclonal antibody, from those cells which have not.

109. The method of claim 107, wherein said monoclonal antibody is anti-LAM1-10 monoclonal antibody.

110. A method of detecting leukocyte activation in an animal, said method comprising

btaining a fluid sample from said animal;

contacting said sample with the monoclonal antibody of claim 103; and

detecting formation of immune complexes formed by said monoclonal antibody, said immune complexes indicating the presence of LAM-1 shed from activated leukocytes.

111. The method of claim 110, wherein said monoclonal antibody is anti-LAM1-10 monoclonal antibody.

112. A method of blocking leukocyte interactions with endothelium in an animal, said method comprising administering an effective amount of the monoclonal antibody or fragment thereof of claim 103 to said animal.

113. The method of claim 112, wherein said monoclonal antibody is anti-LAM1-10 monoclonal antibody.

114. The method of claim 112, wherein said animal is a human.

115. The method of claim 112, wherein said animal is suffering from inflammation.

116. The method of claim 112, wherein said animal is suffering from an autoimmune response.

117. The method of claim 112, wherein said animal is suffering from rejection of an organ or tissue transplant.

118. The method of claim 112, wherein said monoclonal antibody is a chimerized antibody having a variable region derived from anti-LAM1-10 monoclonal antibody and constant regions derived from a human antibody.

119. A method of determining the degree of expression of LAM-1 in a sample of leukocytes, said method comprising

contacting a sample of leukocytes with the antibody of claim 103, and

determining the level of immune complex formation in said sample, said level being indicative of the degree of expression of LAM-1 on said leukocytes.

120. A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-11 monoclonal antibody.

121. The monoclonal antibody of claim 120, wherein said monoclonal antibody is anti-LAM1-11 monoclonal antibody.

122. A hybridoma cell which produces the monoclonal antibody of claim 120.

123. The hybridoma cell line deposited as ATTC #_____.

124. A method of identifying cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with a reagent comprising the monoclonal antibody of claim 120; and

determining which cells form an immune complex with said reagent.

125. A method of isolating cells expressing LAM-1, said meth d comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with the monoclonal antibody of claim 120; and

separating those cells which have formed an immune complex with said monoclonal antibody, from those cells which have not.

126. The method of claim 124, wherein said monoclonal antibody is anti-LAM1-11 monoclonal antibody.

127. A method of detecting leukocyte activation in an animal, said method comprising

obtaining a fluid sample from said animal;

contacting said sample with the monoclonal antibody of claim 120; and

detecting formation of immune complexes formed by said monoclonal antibody, said immune complexes indicating the presence of LAM-1 shed from activated leukocytes.

128. The method of claim 127, wherein said monoclonal antibody is anti-LAM1-11 monoclonal antibody.

129. A method of blocking leukocyte interactions with endothelium in an animal, said method comprising administering an effective amount of the monoclonal antibody or fragment thereof of claim 120 to said animal.

130. The method of claim 129, wherein said monoclonal antibody is anti-LAM1-11 monoclonal antibody.

131. Th method of claim 129, wherein said animal is a human.

132. The method of claim 129, wherein said animal is suffering from inflammation.

133. The method of claim 129, wherein said animal is suffering from an autoimmune response.

134. The method of claim 129, wherein said animal is suffering from rejection of an organ or tissue transplant.

135. The method of claim 131, wherein said monoclonal antibody is a chimerized antibody having a variable region derived from anti-LAM1-11 monoclonal antibody and constant regions derived from a human antibody.

136. A method of determining the degree of expression of LAM-1 in a sample of leukocytes, said method comprising

contacting a sample of leukocytes with the antibody of claim 120, and

determining the level of immune complex formation in said sample, said level being indicative of the degree of expression of LAM-1 on said leukocytes.

137. A monoclonal antibody which recognizes the LAM-1 epitope recognized by anti-LAM1-14 monoclonal antibody.

138. The monoclonal antibody of claim 137, wherein said monoclonal antibody is anti-LAM1-14 monoclonal antibody.

139. A hybridoma cell which produc s the mon cl nal antibody of claim 137.

140. The hybridoma cell line deposited as ATTC #_____.

141. A method of identifying cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with a reagent comprising the monoclonal antibody of claim 137; and

determining which cells form an immune complex with said reagent.

142. A method of isolating cells expressing LAM-1, said method comprising

providing a sample of cells, at least some of which are suspected of expressing LAM-1;

contacting said cells with the monoclonal antibody of claim 137; and

separating those cells which have formed an immune complex with said monoclonal antibody, from those cells which have not.

143. The method of claim 141, wherein said monoclonal antibody is anti-LAM1-14 monoclonal antibody.

144. A method of detecting leukocyte activation in an animal, said method comprising

obtaining a fluid sample from said animal;

contacting said sample with the monoclonal antibody of claim 137; and

detecting formation of immune complexes formed by said monoclonal antibody, said immune complexes indicating the presence of LAM-1 shed from activated leukocytes.

145. The method of claim 144, wherein said monoclonal antibody is anti-LAM1-14 monoclonal antibody.

146. A method of blocking leukocyte interactions with endothelium in an animal, said method comprising administering an effective amount of the monoclonal antibody or fragment thereof of claim 137 to said animal.

147. The method of claim 146, wherein said monoclonal antibody is anti-LAM1-14 monoclonal antibody.

148. The method of claim 146, wherein said animal is a human.

149. The method of claim 146, wherein said animal is suffering from inflammation.

150. The method of claim 146, wherein said animal is suffering from an autoimmune response.

151. The method of claim 146, wherein said animal is suffering from rejection of an organ or tissue transplant.

152. The method of claim 148, wherein said monoclonal antibody is a chimerized antibody having a variable region derived from anti-LAM1-14 monoclonal antibody and constant regions derived from a human antibody.

153. A method of determining the degree of expression of LAM-1 in a sample of leukocytes, said method comprising

contacting a sample of leukocytes with the antibody of claim 137, and

determining the level of immune complex formation in said sample, said level being indicative of the degree of expression of LAM-1 on said leukocytes.

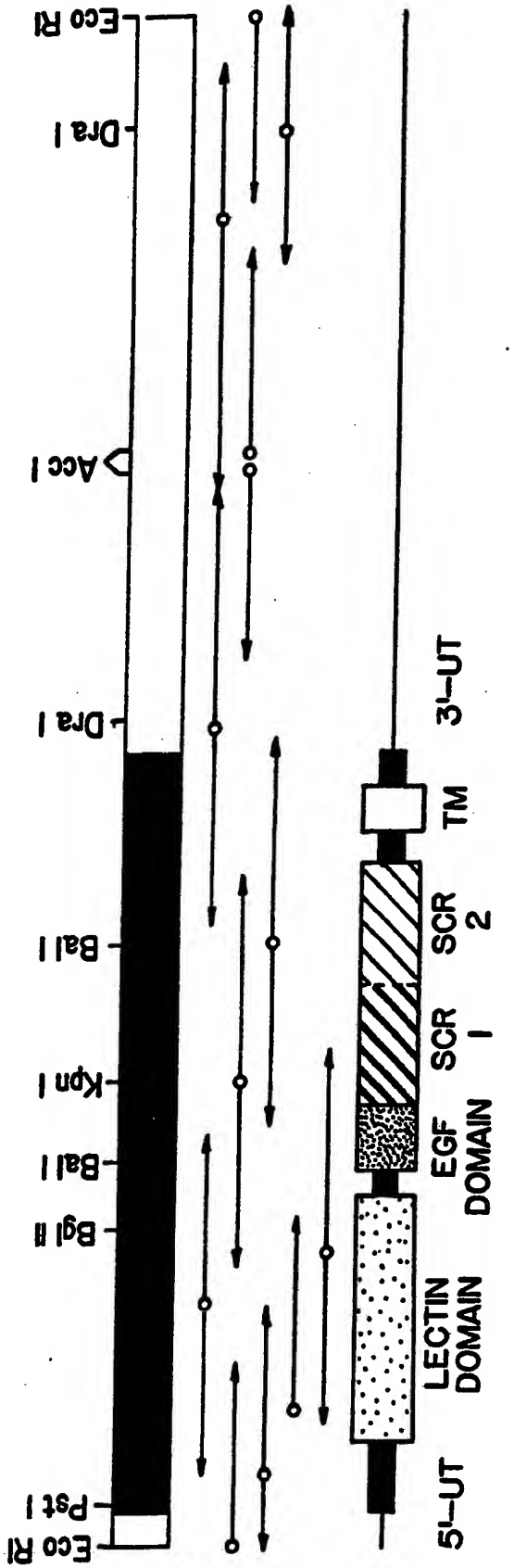
Eco RI
Dra I
Acc I
Dra I
Bal I
Kpn I
Bal I
Bgl II
Pst I
Eco RI
5'-UT
LECTIN DOMAIN
EGF DOMAIN
SCR 1
SCR 2
TM
3'-UT

FIG. IA

FIG. IB

```
                                                                1
                                                                M   G
gaattcCCTTT GGGCAAGGAC CTGAGACCCT TGTGCTAAGTCAAGAGGCTCA ATG GGC
                        10
  C   R   R   T   R   E   G   P   S   K   A   M
TGC AGA AGA ACT AGA GAA GGA CCA AGC AAA GCC ATG    94
                    20
  I   F   P   W   K   C   Q   S   T   Q   R   D   L   W   N   I   30
ATA TTT CCA TGG AAA TGT CAG AGC ACC CAG AGG GAC TTA TGG AAC ATC
                                40
  F   K   L   W   G   W   T   M   L   C   C   D
TTC AAG TTG TGG GGG TGG ACA ATG CTC TGT TGT GAT   168
                        50
  F   L   A   H   H   G   T   D   C ↓ W   T   Y   H   Y   S   E
TTC CTG GCA CAT CAT GGA ACC GAC TGC TGG ACT TAC CAT TAT TCT GAA
        60
  K   P   M   N   W   Q   R   A   R   R   F   C
AAA CCC ATG AAC TGG CAA AGG GCT AGA AGA TTC TGC   262
                    80
  R   D   N   Y   T   D   L   V   A   I   Q   N   K   A   E   I
CGA GAC AAT TAC ACA GAT TTA GTT GCC ATA CAA AAC AAG GCG GAA ATT
            90
  E   Y   L   E   K   T   L   P   F   S   R   S
GAG TAT CTG GAG AAG ACT CTG CCT TTC AGT CGT TCT   346
```

FIG. 2A

```
    100                                         110
Y   Y   W   I   G   I   R   K   I   G   G   I   W   T   W
TAC TAC TGG ATA GGA ATC CGG AAG ATA GGA GGA ATA TGG ACG TGG
                        120
V   G   T   N   K   S   L   T   E   E   A   E   N
GTG GGA ACC AAC AAA TCT CTC ACT GAA GAA GCA GAG AAC  430
            130                                     140
W   G   D   G   E   P   N   N   K   K   N   K   E   D   C
TGG GGA GAT GGT GAG CCC AAC AAC AAG AAG AAC AAG GAG GAC TGC
                                150
V   E   I   Y   I   K   R   N   K   D   A   G   K
GTG GAG ATC TAT ATC AAG AGA AAC AAA GAT GCA GGC AAA  514
                    160
W   N   D   D   A   C   H   K   L   K   A   A   L   C   Y
TGG AAC GAT GAC GCC TGC CAC AAA CTA AAG GCA GCC CTC TGT TAC
                                        180
170
T   A   S   C   Q   P   W   S   C   S   G   H   G
ACA GCT TCT TGC CAG CCC TGG TCA TGC AGT GGC CAT GGA  598
                            190
E   C   V   E   I   I   N   N   Y   T   C   N   C   D   V
GAA TGT GTA GAA ATC ATC AAT AAT TAC ACC TGC AAC TGT GAT GTG
        200                                     210
G   Y   Y   G   P   Q   C   Q   F   V   I   Q   C
GGG TAC TAT GGG CCC CAG TGT CAG TTT GTG ATT CAG TGT  682
                                    220
E   P   L   E   A   P   E   L   G   T   M   D   C   T   H
GAG CCT TTG GAG GCC CCA GAG CTG GGT ACC ATG GAC TGT ACT CAC
                230
P   L   G   N   F   N   F   N   S   Q   C   A   F
CCT TTG GGA AAC TTC AAC TTC AAC TCA CAG TGT GCC TTC  766
    240                                     250
S   C   S   E   G   T   N   L   T   G   I   E   E   T   T
AGC TGC TCT GAA GGA ACA AAC TTA ACT GGG ATT GAA GAA ACC ACC
                        260
C   E   P   F   G   N   W   S   S   P   E   P   T
TGT GAA CCA TTT GGA AAC TGG TCA TCT CCA GAA CCA ACC  850
            270                                     280
C   Q   V   I   Q   C   E   P   L   S   A   P   D   L   G
TGT CAA GTG ATT CAG TGT GAG CCT CTA TCA GCA CCA GAT TTG GGG
                                290
I   M   N   C   S   H   P   L   A   S   F   S   F
ATC ATG AAC TGT AGC CAT CCC CTG GCC AGC TTC AGC TTT  934
                    300
T   S   A   C   T   F   I   C   S   E   G   T   E   L   I
ACC TCT GCA TGT ACC TTC ATC TGC TCA GAA GGA ACT GAG TTA ATT
                                        320
310
G   K   K   K   T   I   C   E   S   S   G   I   W
GGG AAG AAG AAA ACC ATT TGT GAA TCA TCT GGA ATC TGG 1018
                            330
S   N   P   S   P   I   C   Q   K   L   D   K   S   F   S
TCA AAT CCT AGT CCA ATA TGT CAA AAA TTG GAC AAA AGT TTC TCA
                                                350
        340
M   I   K   E   G   D   Y   N   P   L   F   I   P
ATG ATT AAG GAG GGT GAT TAT AAC CCC CTC TTC ATT CCA 1102
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FIG. 2B

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                                                    360
 V   A   V   M   V   T   A   F   S   G   L   A   F   I   I
GTG GCA GTC ATG GTT ACT GCA TTC TCT GGG TTG GCA TTT ATC ATT
                        370
 W   L   A   R   R   L   K   K   G   K   K   S   K
TGG CTG GCA AGG AGA TTA AAA AAA GGC AAG AAA TCC AAG 1186
    380
 R   S   M   N   D   P   Y   *
AGA AGT ATG AAT GAC CCA TAT TAA ATCGCCCTTG GTGAAAGAAA

ATTCTTGGAA TACTAAAAAT CATGAGATCC TTTAAATCCT TCCATGAAAC 1280

GTTTTGTGTG GTGGCACCTC CTACGTCAAA.CATGAAGTGT GTTTCCTTCA

GTGCATCTGG GAAGATTTCT ACCTGACCAA GAGTTCCTTC AGCTTCCATT 1380

TCACCCCTCA TTTATCCCTC AACCCCCAGC CCACAGGTCT TTATACAGCT

CAGCTTTTTC TCTTTTCTGA GGAGAAACAA ATAACACCAT AAAGGGAAAG 1480

GATTCATGTG GAATATAAAG ATGGCTGACT TTGCTCTTTC TTGACTCTTG

TTTTCAGTTT CAATTCAGTG CTGTACTTGA TGACAGACAC TTCTAAATGA 1580

AGTGCAAATT TGATACATAT GTGAATATGG ACTCAGTTTT CTTGCAGATC

AAATTTCGCG TCGTCTTCTG TATACGTCCA GGTACACTCT ATGAAGTCAA 1680

AAGTCTACGC TCTCCTTTCT TTCTAACTCC AGTGAAGTAA TGGGGTCCTG

CTCAAGTTGA AAGAGTCCTA TTTGCACTGT AGCCTCGCCG TCTGTGAATT 1780

GGACCATCCT ATTTAACTGG CTTCAGCCTC CCCACCTTCT TCAGCCACCT

CTCTTTTTCA GTTGGCTGAC TTCCACACCT AGCATCTCAT GAGTGCCAAG 1880

CAAAAGGAGA GAAGAGAGAA ATAGCCTCCG CTGTTTTTTA GTTTGGGGGT

TTTGCTGTTT CCTTTTATGA GACCCATTCC TATTTCTTAT AGTCAATGTT 1980

TCTTTTATCA CGATATTATT AGTAAGAAAA CATCACTGAA ATGCTAGCTG

CAACTGACAT CTCTTTGATG TCATATGGAA GAGTTAAAAC AGGTGGAGAA 2080

ATTCCTTGAT TCACAATGAA ATGCTCTCCT TTCCCCTGCC CCCAGACCTT

TTATCCACTT ACCTAGATTC TACATATTCT TTAAATTTCA TCTCAGGCCT 2180

CCCTCAACCC CACCACTTCT TTTATAACTA GTCCTTTACT AATCCAACCC

ATGATGAGCT CCTCTTCCTG GCTTCTTACT GAAAGGTTAC CCTGTAACAT 2280

GCAATTTTGC ATTTGAATAA AGCCTGCTTT TTAAGTGTTA AAAAgaattc 2330
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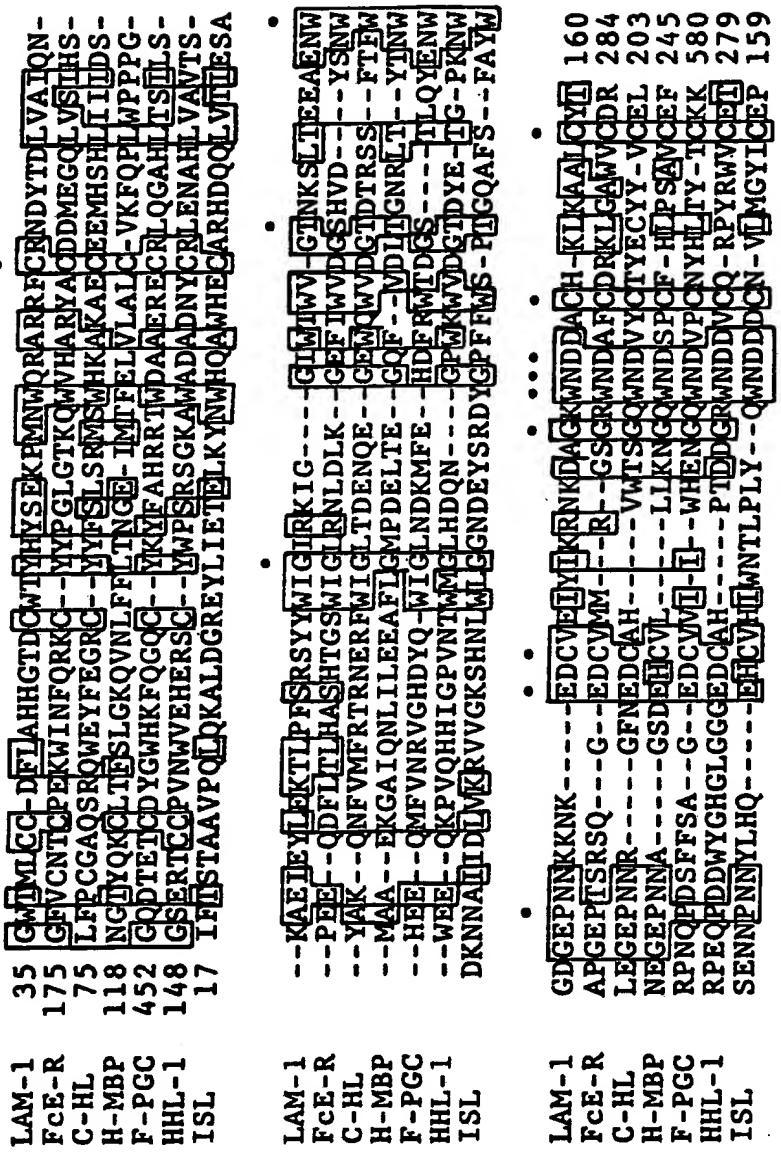
FIG. 2C

FIG. 3A


LAM-1 35
FcE-R 175
C-HL 75
H-MBP 118
F-PGC 452
HHL-1 148
ISL 17
LAM-1
FcE-R
C-HL
H-MBP
F-PGC
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FcE-R 284
C-HL 203
H-MBP 245
F-PGC 580
HHL-1 279
ISL 159

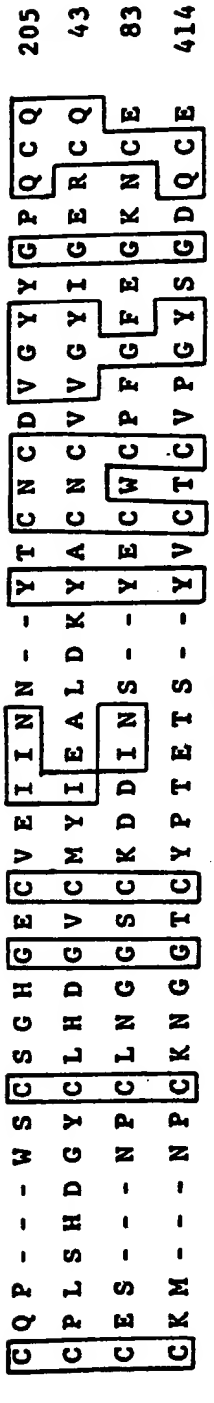

LAM-1 173 C Q P - - - W S C S G H G E C V E I I N N - - Y T C N C D V G Y Y G P Q C Q 205
EGF 6 C P L S H D G Y C L H D G V C M Y I E A L D K Y A C N C V V G Y I G E R C Q 43
F-IX 51 C E S - - - - N P C L N G G S C K D D I N S - - Y E C W C P F G F E G K N C E 83
F-PGCP 382 C K M - - - - N P C K N G G T C Y P T E T S - - Y V C T C V P G Y S G D Q C E 414

FIG. 3B

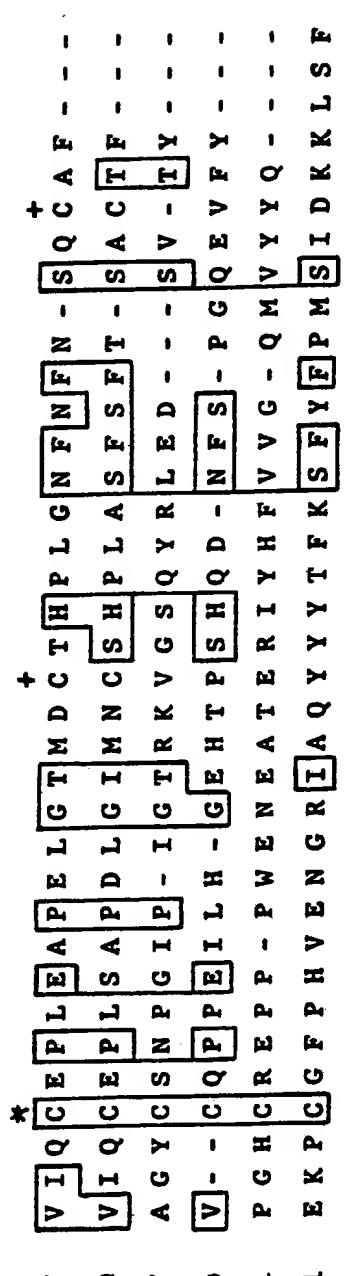
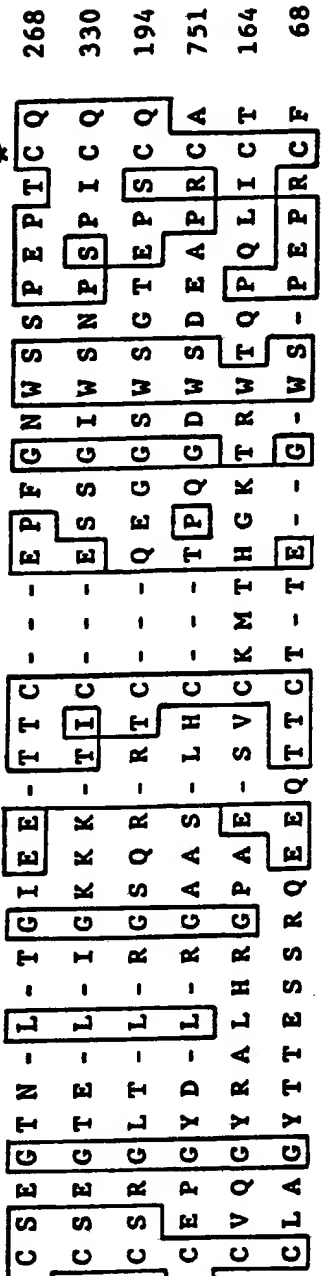

LAM-1 207 V I Q C E P L E A P E L G T M D C T H P L G N F N F N - S Q C A F - - -
LAM-1 269 V I Q C E P L S A P D L G I M N C S H P L A S F S F T - S A C T F - - -
Ba 137 A G Y C S N P G I P - I G T R K V G S Q Y R L E D - - - S V - T Y - - -
CR1 694 V - - C Q P P E I L H - G E H T P S H Q D - N F S - P G Q E V F Y - - -
IL-2R 101 P G H C R E P P - P W E N E A T E R I Y H F V V G - Q M V Y Y Q - - - -
F-XIII 1 E K P C G F P H V E N G R I A Q Y Y Y T F K S F Y F P M S I D K K L S F

FIG. 3C


LAM-1 S C S E G T N - L - T G I E E - T T C - - - E P F G N W S S P E P T C Q 268
LAM-1 I C S E G T E - L - I G K K K - T I C - - - E S S G I W S N P S P I C Q 330
Ba H C S R G L T - L - R G S Q R - R T C - - - Q E G G S W S G T E P S C Q 194
CR1 S C E P G Y D - L - R G A A S - L H C - - - T P Q G D W S D E A P R C A 751
IL-2R - C V Q G Y R A L H R G P A E - S V C K M T H G K T R W T Q P Q L I C T 164
F-XIII F C L A G Y T T E S S R Q E E Q T T C T - T E - - G - W S - P E P R C F 68

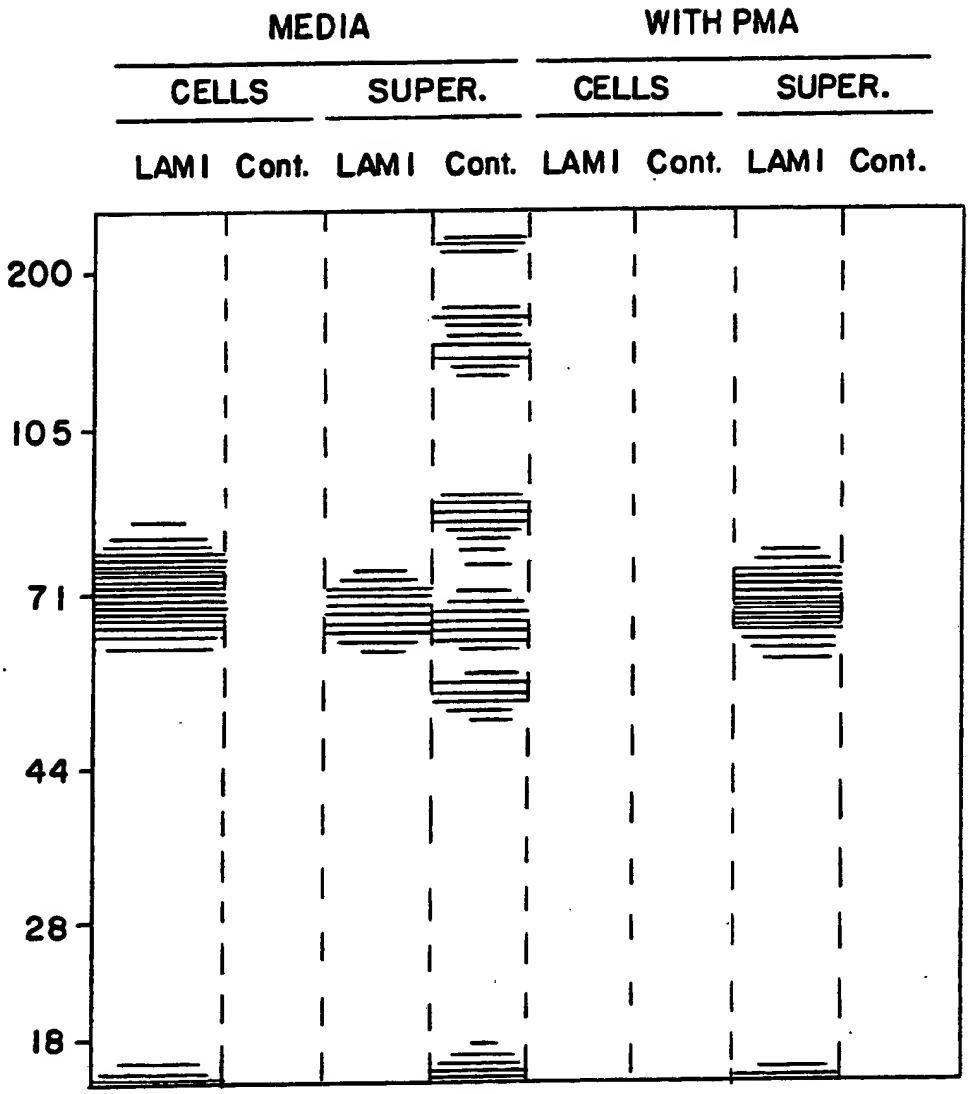

MEDIA
WITH PMA
CELLS
SUPER.
CELLS
SUPER.
LAMI Cont. LAMI Cont. LAMI Cont. LAMI Cont.
200
105
71
44
28
18

FIG. 4

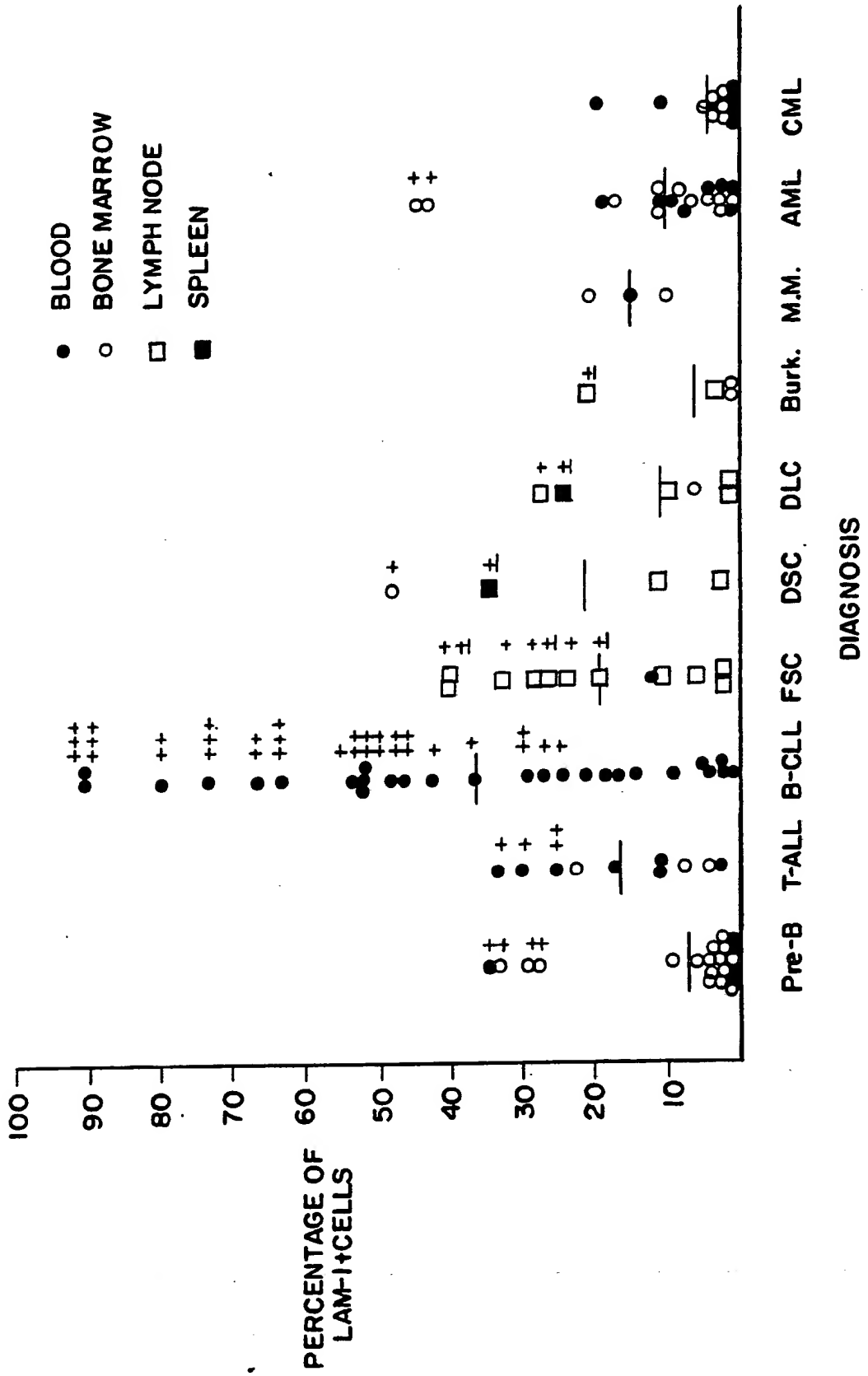

BLOOD
BONE MARROW
LYMPH NODE
SPLEEN
PERCENTAGE OF LAM-1+CELLS
100
90
80
70
60
50
40
30
20
10
Pre-B
T-ALL
B-CLL
FSC
DSC
DLC
Burk.
M.M.
AML
CML
DIAGNOSIS

FIG. 5

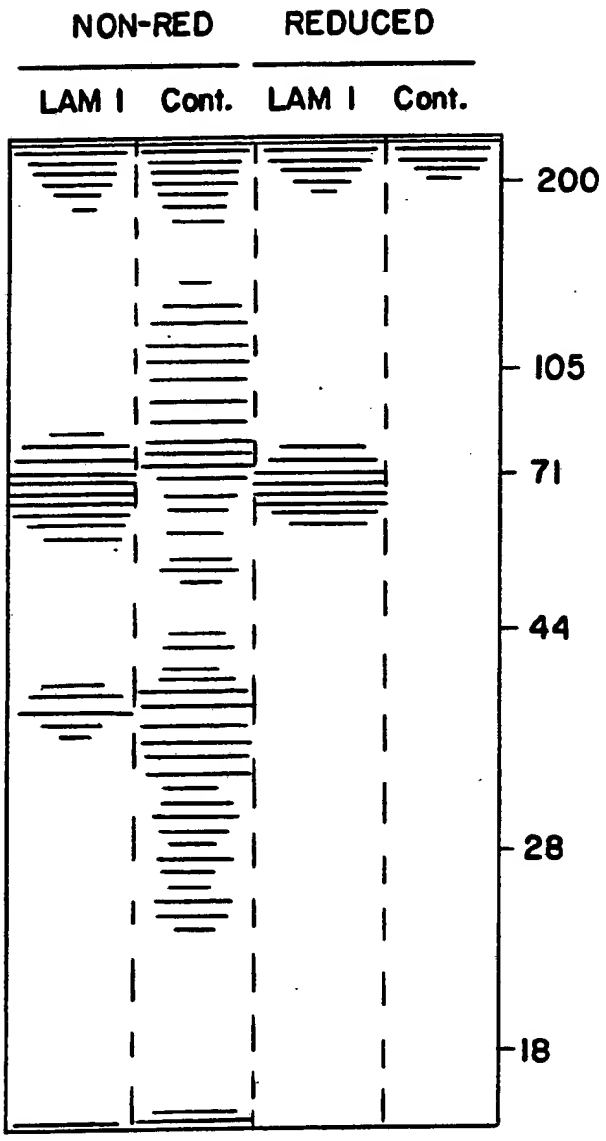


FIG. 6

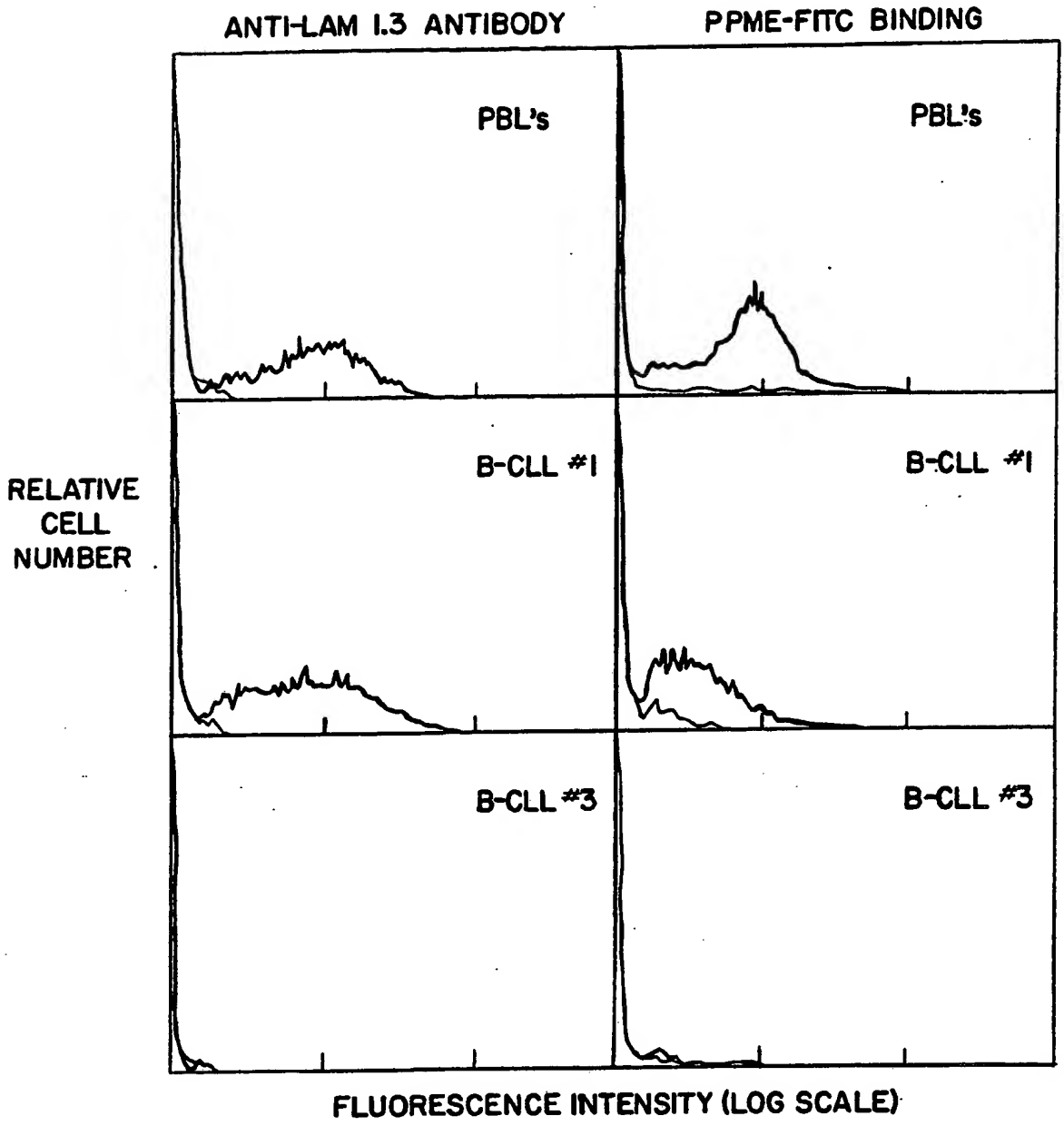

ANTI-LAM 1.3 ANTIBODY
PPME-FITC BINDING
PBL's
PBL's
B-CLL #1
B-CLL #1
B-CLL #3
B-CLL #3
RELATIVE CELL NUMBER
FLUORESCENCE INTENSITY (LOG SCALE)

FIG. 7

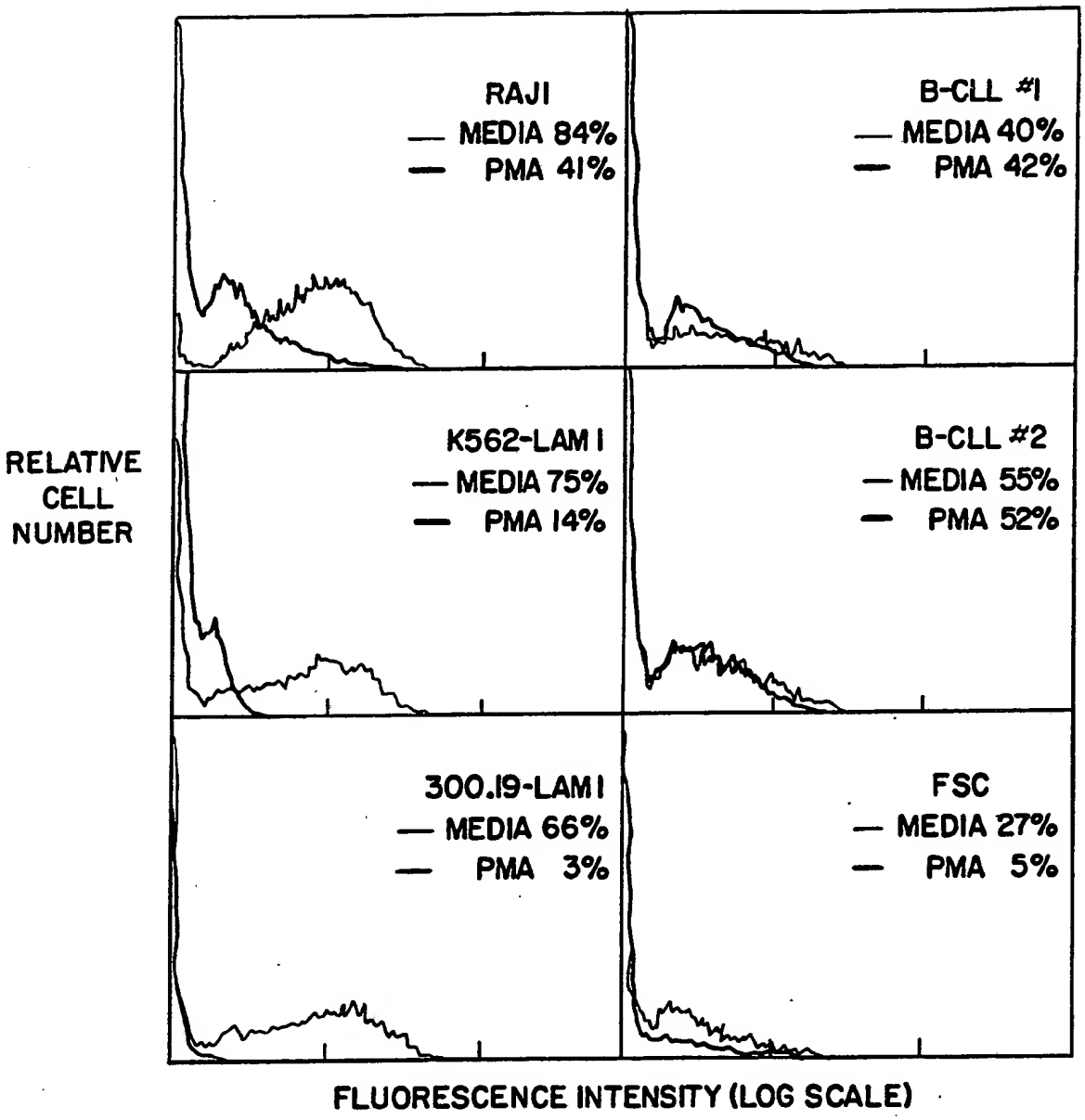

RAJI
— MEDIA 84%
— PMA 41%
B-CLL #1
— MEDIA 40%
— PMA 42%
K562-LAM I
— MEDIA 75%
— PMA 14%
B-CLL #2
— MEDIA 55%
— PMA 52%
300.19-LAM I
— MEDIA 66%
— PMA 3%
FSC
— MEDIA 27%
— PMA 5%
RELATIVE CELL NUMBER
FLUORESCENCE INTENSITY (LOG SCALE)

FIG. 8

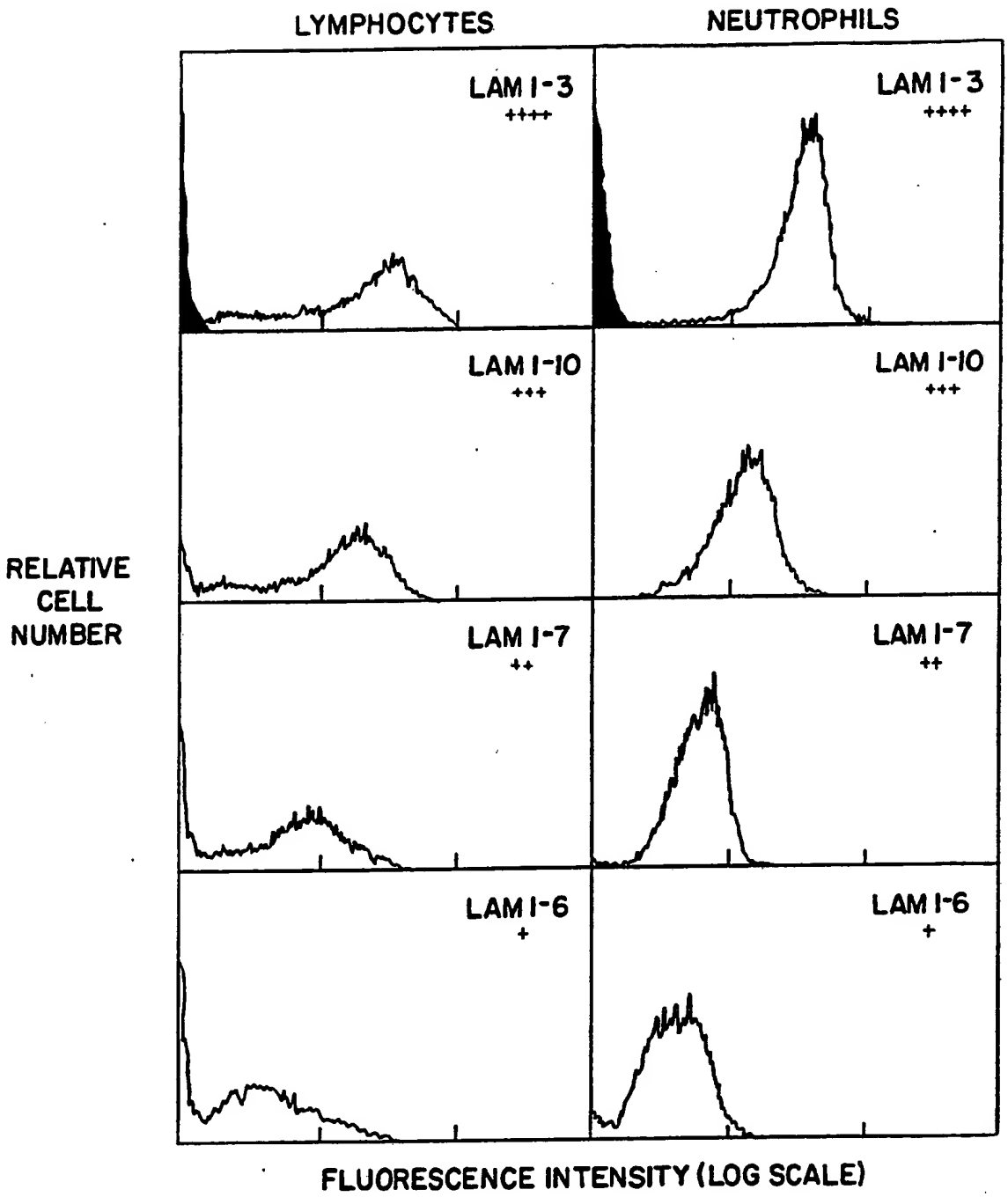

LYMPHOCYTES
NEUTROPHILS
LAM I-3
++++
LAM I-3
++++
LAM I-10
+++
LAM I-10
+++
LAM I-7
++
LAM I-7
++
LAM I-6
+
LAM I-6
+
RELATIVE CELL NUMBER
FLUORESCENCE INTENSITY (LOG SCALE)

FIG. 9

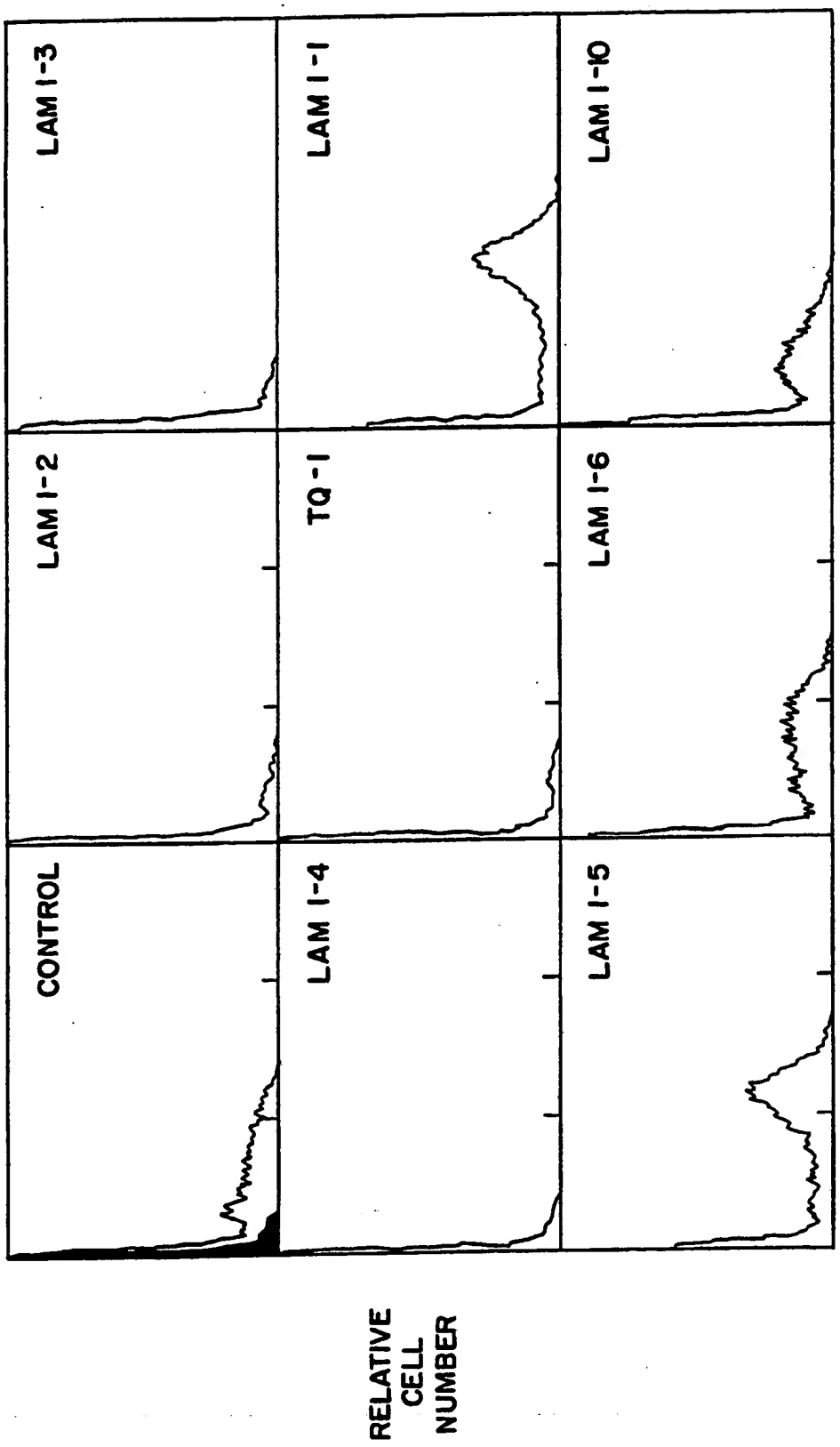

LAM I-3
LAM I-1
LAM I-10
LAM I-2
TQ-1
LAM I-6
CONTROL
LAM I-4
LAM I-5
RELATIVE CELL NUMBER
FLUORESCENCE INTENSITY

FIG. 10

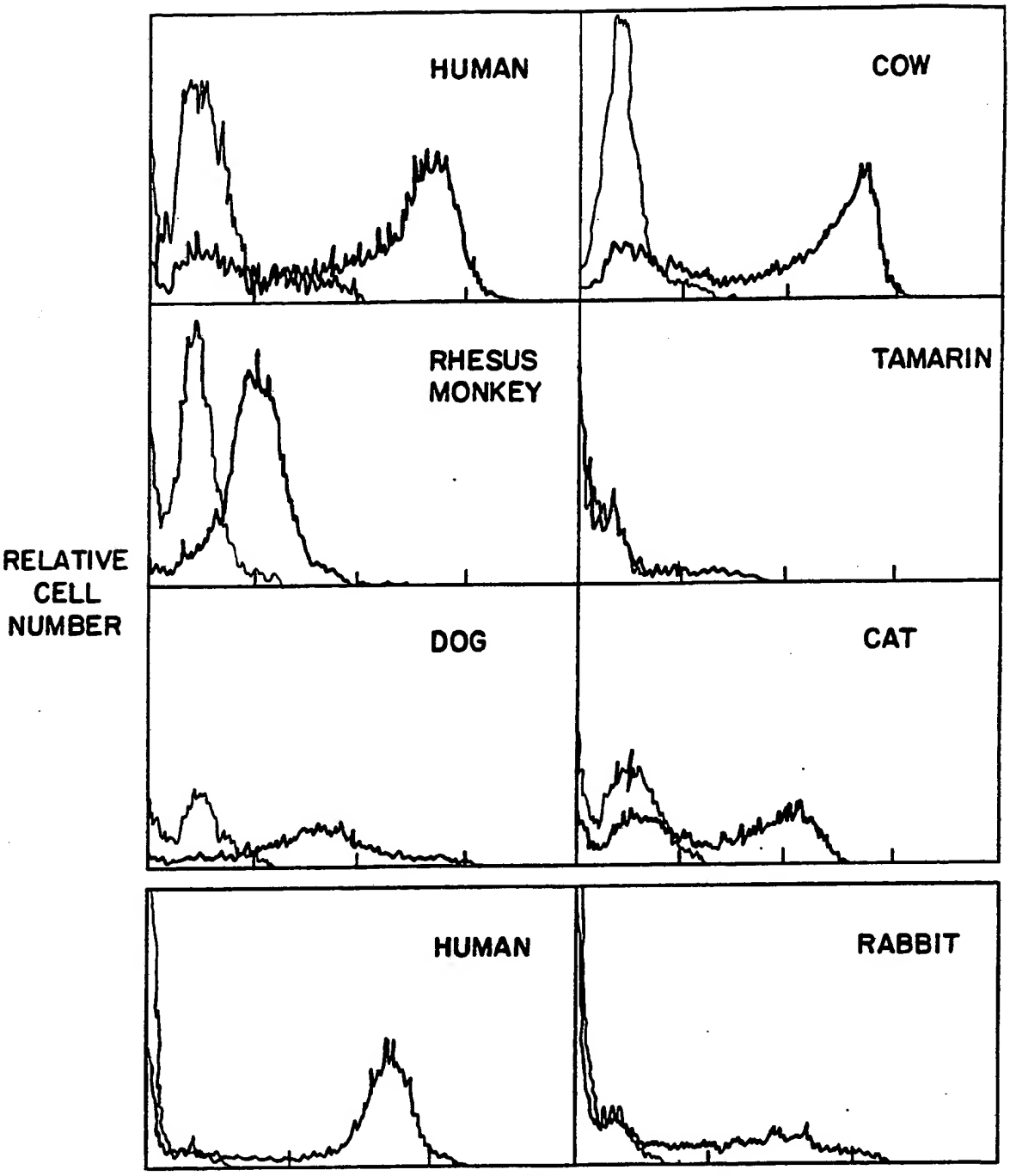

HUMAN
COW
RHESUS MONKEY
TAMARIN
DOG
CAT
HUMAN
RABBIT
RELATIVE CELL NUMBER
FLUORESCENCE INTENSITY

FIG. 11

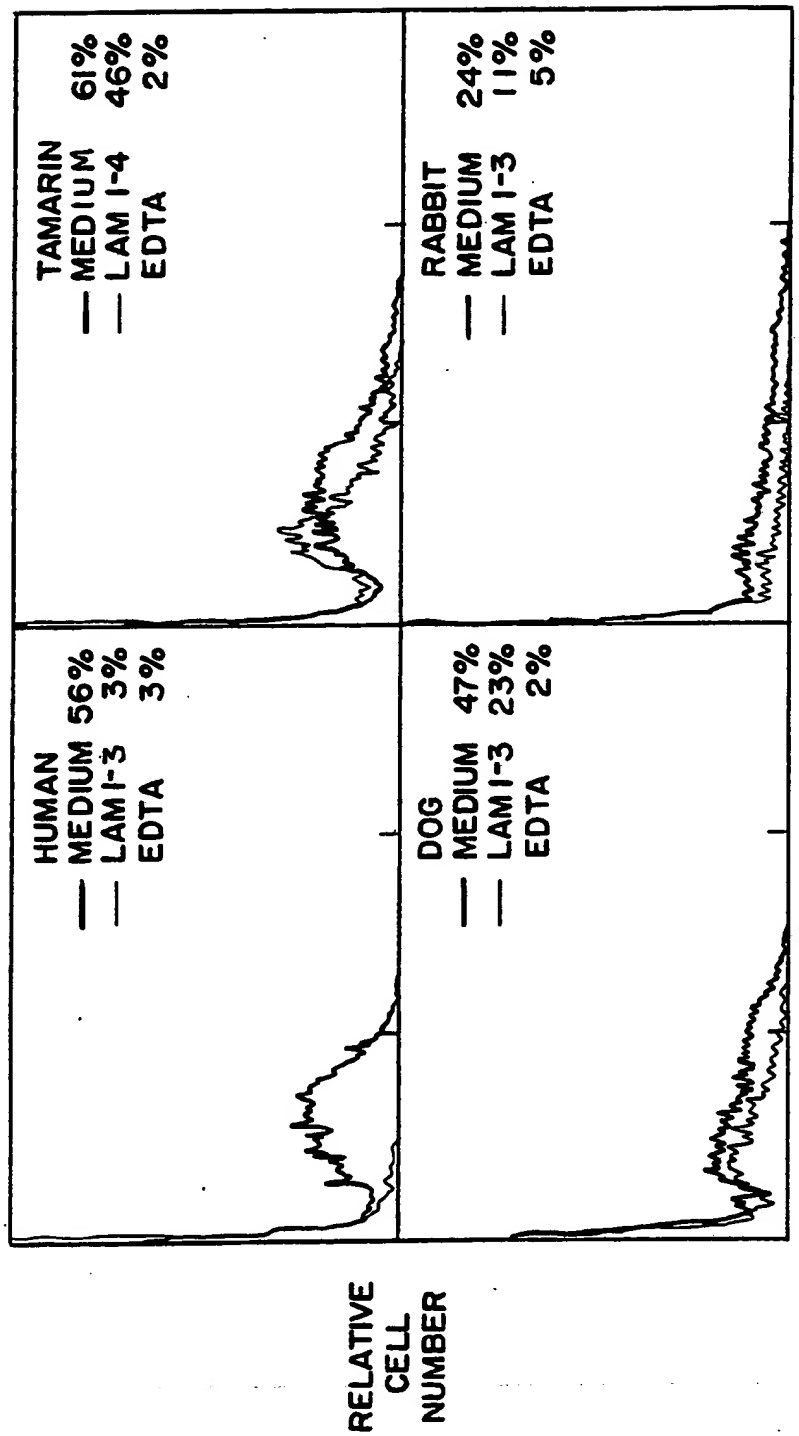

TAMARIN
MEDIUM 61%
LAM I-4 46%
EDTA 2%
RABBIT
MEDIUM 24%
LAM I-3 11%
EDTA 5%
HUMAN
MEDIUM 56%
LAM I-3 3%
EDTA 3%
DOG
MEDIUM 47%
LAM I-3 23%
EDTA 2%
FLUORESCENCE INTENSITY
RELATIVE CELL NUMBER

FIG. 12

FUNCTIONAL AND STRUCTURAL REGIONS OF LAM-I
IDENTIFIED BY MONOCLONAL ANTIBODIES

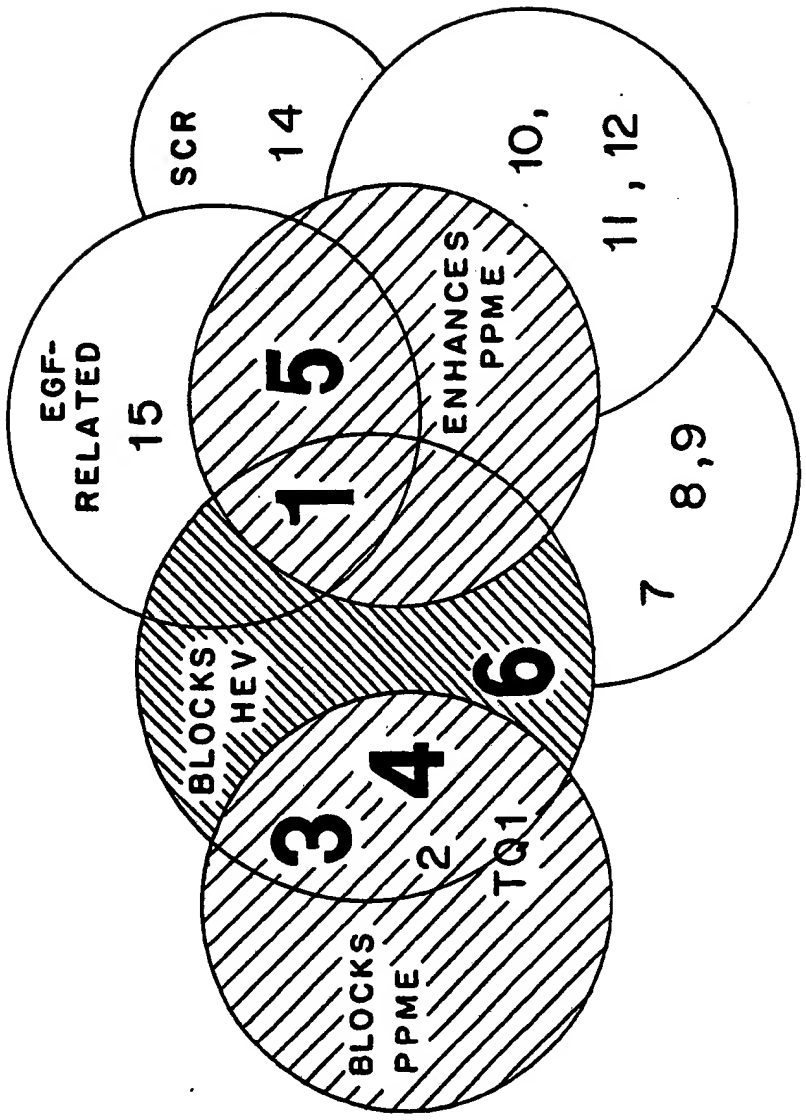


FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/06127

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/04, 39/00; C07K 13/00, 15/28; C12N 15/00, C12P 21/08
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8; 435/70.21, 172.2; 530/387.1, 388.1, 388.22, 388.24, 388.85, 866, 867

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, SCISEARCH, WPI, APS
SEARCH TERMS: LAM 1, ANTIBODY, TEDDER

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF IMMUNOLOGY, Volume 128, No. 1, issued 01 January 1982, E.L. Reinherz et al., "Heterogeneity of Human T4+ Inducer T cells Defined by a Monoclonal Antibody that Delineates Two Functional Subpopulations", pages 463-468, see entire document.	1-153
Y	PROC. NATL. ACAD. SCI. USA, Volume 87, issued March 1990, Kishimoto et al, "Identification of a Human Peripheral Lymph Node Homing Receptor: A Rapidly Down-Regulated Adhesion Molecule", pages 2244-2248, see entire document.	1-153
Y	JOURNAL OF CELL BIOLOGY, Volume 107, issued November 1988, N.W. Wu et al., "Evolutionary Conservation of Tissue-specific Lymphocyte-Endothelial Cell Recognition Mechanisms Involved in Lymphocyte Homing", pages 1845-1851, entire document.	1-153
Y	JOURNAL OF IMMUNOLOGY, Volume 144, issued 15 January 1990, T.F. Tedder, "Expression of the Human Leukocyte Adhesion Molecule, LAM1: Identity with the TQ1 and Leu-8 Differentiation Antigens", pages 532-540, see entire document.	1-153

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
04 SEPTEMBER 1992

Date of mailing of the international search report
16 SEP 1992

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/06127

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Volume 170, issued 01 July 1989, T.F. Tedder et al., "Isolation and Chromosomal Localization of cDNAs Encoding a Novel Human Lymphocyte Cell Surface Molecule, LAM-1", pages 123-133, see entire document.	1-153
Y	JOURNAL OF CELL BIOLOGY, Volume 109, issued July 1989, B.R. Bowen et al., "Characterization of a Human Homologue of the Murine Peripheral Lymph Node Homing Receptor", pages 421-427, see entire document.	1-153
Y	USA 5,098,833 (Lasky et al.) 24 March 1992, see entire document.	1-153
Y	CELL, Volume 56, issued 24 March 1989, Stoolman, "Adhesion Molecules Controlling Lymphocyte Migration", pages 907-910, see entire document.	1-153
Y	CELL, Volume 62, issued 13 July 1990, Osborn, "Leukocyte Adhesion to Endothelium in Inflammation", pages 3-6, entire document.	1-153

International application No.
PCT/US92/06127

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.8; 435/70.21, 172.2; 530/387.1, 388.1, 388.22, 388.24, 388.85, 866, 867